

MOLECULAR IMMUNOLOGY OF THE RAT COLON CARCINOMA

Methodology for analysis of tumor
associated antibodies and antigens



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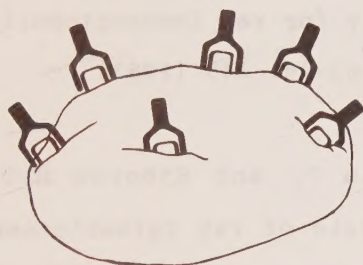
University of Lund

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This dissertation is based on the following publications:

- I Nilsson R, Myhre E, Kronvall G, and Sjögren H.O.
Fractionation of rat IgG subclasses and screening for
IgG F_C-binding to bacteria.
Molec.Immun. 19:119-126 (1982)
- II Nilsson R, Myhre E, Kronvall G, and Sjögren H.O.
Different Protein-A immunosorbents may have different
binding specificity for rat immunoglobulins.
J.Immunol.Methods 62:241-245 (1983)
- III Nilsson R, Brodin T, and Sjögren H.O.
Quantitative analysis of rat Ig(sub)classes binding to
cell surface antigens.
J.Immunol.Methods 55:179-191 (1982)
- IV Nilsson R and Sjögren H.O.
Antigen-independent binding of rat immunoglobulins in a
radioimmunoassay. Solutions to an unusual background
problem.
J.Immunol.Methods 66:17-25 (1984)
- V Nilsson R, Jansson B, Brodin T, and Sjögren H.O.
Use of butanol-extracted antigens of rat colon
carcinoma for analysis of humoral tumor immunity.
Cancer Res. (submitted for publication)

Nilsson R, Jansson B, and Sjögren H.O.

Partial biochemical characterization of 1-butanol extractable rat colon carcinoma antigens defined by syngeneic antibodies.

Cancer Res. (submitted for publication)

These publications will be referred to by their Roman numerals. The summary also includes some unpublished results.

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ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
BETA	butanol-extractable tumor antigens
BN	Brown Norway rat (RT-1 ⁿ)
CBE	crude butanol extract
CdL	complement dependent lysis
FcR	Ig-binding molecules
FCS	fetal calf serum
Ig	immunoglobulin
LAI	leukocyte adherence inhibition assay
NRS	normal reference serum
PBS	phosphate buffered saline
RIA	radio immunoassay
SBF	serum blocking factor
SpA	<u>Staphylococcus aureus</u> protein-A
SpG	human group G streptococcal protein-G
WF	Wistar/Furth rat (RT-1 ^u)

INTRODUCTION

The immune response has traditionally been divided into two well separated parts, humoral immunity and cellular immunity. Humoral immunity was defined as immunity that could be transferred from an immune donor to a non-immune recipient by the non-cellular part of the blood, i.e. by serum or plasma. Cellular immunity could be transferred by the blood cells. The immunochemists, who studied the humoral part, concentrated most of their work on biochemical studies of immunoglobulins, i.e. purification and characterization of Ig, precipitation mechanisms and other use of Ig in detection of other molecules, often without direct connection with the immune reactions. The immunobiologists studied the immune responses by use of in vivo experiments or biological tests of cell activities in vitro, often by use of undefined cell populations and serum. This resulted in description of immunological reactions as "phenomena", "activities" and "factors".

In modern immunology, it is not meaningful to make such divisions of the immune response, as it is recognized to involve complex, totally integrated mechanisms of both cellular and humoral components. The molecular immunologists work on biochemical and immunobiological characterization of the molecules, free or cell-bound, and of the molecular reactions involved in the complete immune response against foreign materials.



1. Why studies of immunoglobulins?

The immunoglobulins were among the first immunological components shown to have specific affinity for the foreign molecules (antigens) inducing the immune reaction. Immunoglobulins have been shown to be involved in a variety of immunological reactions. Some antibodies against cellular antigens mediate cytolytic activities by activation of the complement cascade (CdL, complement dependent cytotoxicity) or certain special types of leukocytes (ADCC, antibody dependent cellular cytotoxicity). Antibody binding to parasites or microorganisms facilitates their elimination by phagocytosis. Immunoglobulins are involved in the regulation of the immune response, as idiotypic antibodies in the idiotypic network or combined with antigen in immune complexes which activate regulatory T-lymphocytes. The immunoglobulins are, however, a very diverse family of molecules with eight different classes or subclasses, each having different properties and functions. Most studies of Ig-functions have been performed with unseparated serum or whole Ig-preparations without analysis of the content of the different (sub)classes. As immunoglobulins are very important components of the immune response having a variety of functions, it is important to elucidate further their role and the functions and involvements of different classes and subclasses.

2. Why studies on rats?

It may not be directly obvious why time and effort should be spent on studies on rats and rat

immunoglobulins. The rat is, however, one of the most widely used experimental animals in immunology, especially for studies concerning immunoglobulin functions. For understanding of e.g. allergy, transplantation immunology, immune responses against tumors or pathogens and autoimmunity, important contributions have come from experimental models utilizing rats. In serological studies the use of rats has great advantages, compared to the use of the most common experimental animal - the mouse. This is because sufficient volumes of serum can be obtained repeatedly from individual animals without affecting the general condition of the animal.

The interest in rat immunoglobulins has also increased by the evolution of the hybridoma technology for production of monoclonal antibodies. Most investigators use mouse myeloma lines to construct their hybridomas. However, it has recently been claimed that the use of rat x rat hybridomas may have advantages over their mouse counterparts (1,2). This will require rapid and efficient methods for purification and analysis of rat immunoglobulins.

3. Why serological studies of tumor immunity?

The immune response against tumors is dualistic, i.e. it can help the host to reject the tumor or it can facilitate the growth and spread of the tumor. An understanding of the mechanisms behind these two types of response is of course of great importance. The

immunoglobulins are involved on both sides. They can cause a cytotoxic effect on cells by the CdL and ADCC mechanisms or they can facilitate the tumor cell growth when, in the form of immune complexes, they activate the suppressive arm of cellular response leading to inhibition of cytotoxic T-lymphocytes and other effector cells. It may be assumed, that immunoglobulins mediating these different functions belong to different (sub)classes. The relative importance of immunoglobulin-mediated functions compared to other effector mechanisms in rejection or enhancement of tumor growth is not known.

The analysis of different immunoglobulin (sub)classes reactive against tumors is of great importance for the development of immunodiagnosis and for establishment of methods of immunotherapy in cancer patients.

4. Aims and outline of the present investigation.

The aims of this study were to:

- * Establish fractionation methods for rat IgG subclasses
- * Produce monospecific antisera against rat IgG(sub)classes
- * Establish an amplified immunoassay for analysis of antibodies binding to cell surface antigens
- * Develop sensitive tests for analysis of antibody specificity
- * Study the biochemical properties of tumor-associated antigens.

Outline of this study:

- * Methods for effective fractionation of rat IgG-subclasses were developed and the potential use of new bacteriosorbents in affinity techniques for rat Ig was analyzed (I, II)
- * Monospecific rabbit antisera against rat Ig(sub)classes were produced and a radioimmunoassay of antigen binding antibodies was established and evaluated (III, IV)
- * Preparation methods for cell surface molecules functional as target antigens in a RIA for tumor-reactive antibodies were developed (V)
- * Two sensitive tests for evaluation of binding specificities of tumor-reactive serum were established (V)
- * A biochemical characterization of rat colon carcinoma associated antigens was initiated (VI).

1. Rat immunoglobulins.

A majority of the fundamental knowledge about rat immunoglobulins is based on work by Herve Bazin and his coworkers and their studies on monoclonal Ig produced by transplantable immunoglobulin-secreting tumors (ileocoecal immunocytomas) in rats (8,9). After analysis of a great number of monoclonal immunoglobulins, rat Ig could be divided in classes and subclasses and the nomenclature proposed by Bazin et al, 1974 (3), is now generally established. Five different classes of immunoglobulins are recognized in the rat with designations: IgG, IgM, IgA, IgD, and IgE. The IgG class contains four different subclasses: IgG1, IgG2a, IgG2b, and IgG2c. Serum concentrations and some other properties of these different immunoglobulins are summarized in Table 1. The IgD is present mainly on the cell surface of B-lymphocytes. Therefore this class is not included in the studies of serum Ig. IgE is mainly involved in hypersensitivity reactions and is not further studied in the work presented in this thesis.

The Ig(sub)classes have different physiochemical properties, which are used as a basis for various separation methods. However, the differences between the classes, and especially the subclasses, are very minute and combinations of different sophisticated fractionation methods are needed for efficient purification. The most widely used methods are ion exchange chromatography, gel filtration, and affinity

Table 1: Rat immunoglobulins

	IgG1	IgG2a	IgG2b	IgG2c	IgM	IgA	IgE	IgD
Serum concentration (mg/ml) ¹	5,85	8,00	N.T.	2,60	0,56	0,13	0,02 - ⁶	0
Serum concentration ₂ (mg/ml) ²	11,0	11,0	1,3	2,2	0,97	0,15	10	0,008
Affinity for protein-A ³	+	-	+	+	-	-	N.T.	N.T.
Affinity for protein-A ⁴	+	-	-	+	<u>+</u>	<u>+</u>	-	N.T.
Affinity for protein-G ⁵	+	+	+	+	-	-	N.T.	N.T.
Activation of rat complement								
classical pathway ⁶	+	+	+	+	- ⁷	N.T.	N.T.	N.T.
alternative pathway ⁶	-	-	+	-	N.T.	N.T.	N.T.	N.T.

N.T. : not tested or information not given

- 1: ref. 3
- 2: ref. 4
- 3: paper I, II
- 4: ref. 5
- 5: paper I and this thesis
- 6: ref. 6
- 7: ref. 7

chromatography with Protein-A. Protein-A, a cell wall component of Staphylococcus aureus bacteria, binds to the Fc-part of some immunoglobulins (10). This property has been demonstrated with immunoglobulins of several mammalian species (11) and has proved to be useful for affinity chromatography of immunoglobulins (12, 15). Binding of the Fab-part of some immunoglobulins to Protein-A has also been reported (13). It has been claimed in numerous publications that rat immunoglobulins do not bind to Protein-A (16) or bind with very low affinity (10,15). As will be discussed below, however, Protein-A affinity chromatography has been used for successful fractionation of three of the four IgG subclasses. These discrepancies may have different reasons. The standard procedure for preparation of IgG-fractions by DEAE-chromatography, originally developed for rabbit IgG (14), will result in an IgG-fraction containing only IgG2a. The other subclasses may be missed even if an anti-IgG serum is used to control the separation, as IgG2a has a very dominating immunogenicity in rabbits resulting in antisera recognizing mainly IgG2a. When whole serum is used in competitive binding inhibition experiments of Ig binding to Protein-A, the reactivity may be missed, as the low affinity of rat IgG for Protein-A will require high concentrations of rat IgG in order to inhibit the binding of human or rabbit IgG to Protein-A.

2. Purification of IgG1 and IgG2c.

The fractionation procedures established for the purification of rat Ig are summarized in figure 1. At

PURIFICATION OF RAT IMMUNOGLOBULINS

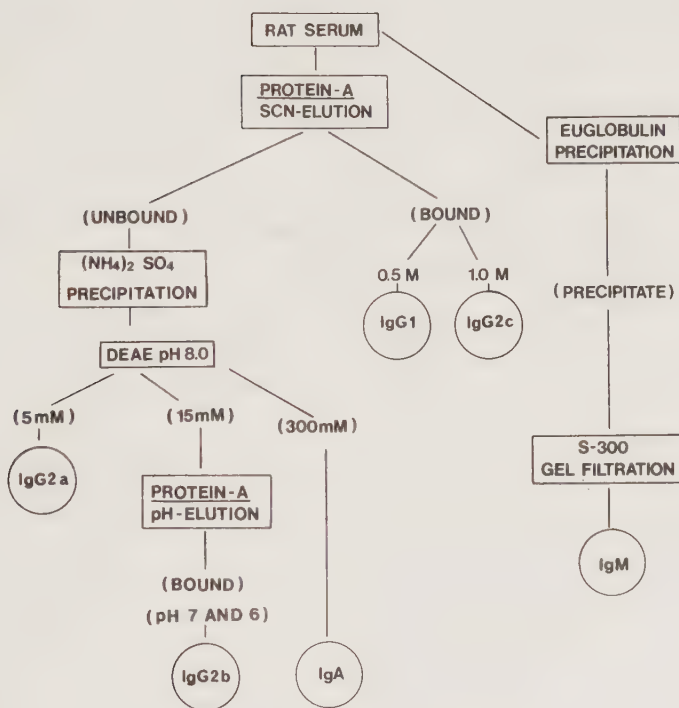


Figure 1 :

Flow chart for purification of different rat immunoglobulin (sub)classes from rat serum by Protein-A-affinity chromatography with SCN- and pH-elution, DEAE-ion exchange chromatography, and gelfiltration.

the time when this investigation was initiated, it had been reported that only IgG1 and IgG2c bind to Staphylococcus aureus Protein-A (SpA) (5). At that time the standard procedure for affinity chromatography on SpA-Sepharose was a simple on-off procedure i.e. the sample was applied, the column was washed and bound material was eluted with a low pH-buffer (17). When I utilized this method, an effluent free of IgG1 and IgG2c but containing the other immunoglobulins was obtained. The eluate contained IgG1, IgG2c, and IgG2b. In order to investigate whether the bound IgG subclasses have different affinity for SpA and to improve the fractionation, a linear sodium thiocyanate gradient between 0 and 3M NaSCN was used for elution. The result was very successful as two base-line separated peaks were obtained, the first at about 0.3M NaSCN containing mainly IgG1 with some IgG2b, and the second peak at about 0.7M NaSCN in which only IgG2c could be detected. The fractionation is simplified by the use of a step-wise elution instead of the gradient elution, IgG1 being eluted with 0.5M NaSCN followed by elution of IgG2c with 1.0M NaSCN. Contaminating IgG2b is eliminated by a wash with PBS+1M NaCl pH 6.0 before elution with NaSCN (Paper I).

The denaturation effect of NaSCN is greatly inhibited by addition of 0.1M glycine to the buffers (18). Without addition of glycine, a great portion of the IgG will precipitate if the fractions are not immediately applied to gel filtration, while over night dialysis of the fractions can be performed without precipitation if glycine is included.

This potential of using SpA-Sepharose for fractionation of IgG1 and IgG2c from normal serum has been confirmed by Rousseaux et al (19). They established the same procedure with SCN-elution, but reported detectable amounts of IgG2c in the 0.5M NaSCN eluate. They have not included the washing step with 1.0M NaCl pH 6.0 buffer and do not report the presence of IgG2b in the SCN-fractions. They also evaluated the stepwise pH-elution method described for mouse IgG (20) and recommended the use of pH-elution for fractionation of IgG1 and IgG2c. This elution method did not function well in my hands, resulting in poor separation. In conclusion, IgG1 and IgG2c can be separated by a rather mild technique without any pretreatment of the serum by the use of SpA-affinity chromatography with SCN-elution.

3. Purification of IgG2a.

Special properties of IgG2a are the low affinity for DEAE-ion exchange matrices at neutral pH and the lack of binding to Protein-A. The method described by Medgyesi et al (5) was used for purification, but ammonium sulphate precipitation was used instead of the euglobulin precipitation. The immunoglobulin fraction of serum was precipitated by addition of saturated ammonium sulphate to a final saturation of 40%. The precipitate was dissolved and dialyzed against 5mM sodium phosphate buffer pH 8.0 and then applied to a column of DEAE-cellulose equilibrated with this buffer. The effluent with 5mM phosphate buffer pH 8.0 contained pure IgG2a

without contamination of other immunoglobulins or serum proteins (paper I). This method is very similar to the procedure originally described for rabbit-IgG (14) and then used as a standard procedure for preparation of IgG-fractions of serum from various species. Therefore, many studies on rat IgG have been performed with pure IgG2a instead of the assumed total rat IgG-fraction (21).

4. Purification of IgG2b.

IgG2b has been a problem in studies on rat IgG subclasses, as no fractionation method has been available for its purification. The electrophoretic properties of IgG2b are very similar to that of IgG2a, and therefore IgG2b and IgG2a mostly cofractionate during ion exchange chromatography. When this work was initiated the best result was obtained by chromatography on DEAE-cellulose (5). After elution of IgG2a with 5mM phosphate buffer pH 8.0 a mixture of IgG2a and IgG2b could be eluted with 15mM phosphate buffer pH 8.0 (5, paper I). For evaluation of IgG2b functions, comparisons between this IgG2a / IgG2b mixture and pure IgG2a had to be performed.

When I evaluated the efficiency of the stepwise pH-elution method for Ig bound to SpA-Sepharose at pH 8.0, described for mouse IgG (20), for elution of rat IgG, I noticed that pure IgG2b was obtained in the pH 7 and pH 6 eluates (paper I). This result was not in accordance with the data presented by Medgyesi et al (5) that IgG2b does not interact with Protein-A. The result

was confirmed in repeated experiments, however, and the procedure was used for fractionation of IgG2b. It has also been shown to be possible to use untreated serum applied to SpA-Sepharose at pH 8.0 for preparation of IgG2b. Therefore it is possible to obtain pure fractions of three of the four subclasses with a single affinity chromatographic procedure utilizing untreated rat serum.

Discrepancies between the Ig-binding properties of different Protein-A matrices were demonstrated in experiments on the binding of radio-labeled fractionated rat IgG subclasses to S. aureus bacteria and different types of Protein-A-Sepharoses (paper II). The Protein-A-Sepharose 4B CL displayed a binding of IgG2b more than twice higher than that of S. aureus bacteria. This difference was even more pronounced when the experiment was performed at pH 8 instead of pH 7.3, i.e. the binding to SpA-Sepharose 4B CL was 3-4 times higher than the binding to S. aureus. Rousseaux et al have also reported that IgG2b has affinity for SpA-Sepharose (19). However, they only obtained a slight retardation of IgG2b in the effluent from SpA-Sepharose at pH 8.0 and all IgG2b was detected in the pH 8-fraction. One of the authors has used the procedure described above, however, for isolation of pure IgG2b by use of SpA-Sepharose (22, 23).

5. Purification of IgM and IgA.

IgM and IgA were purified from normal rat serum by a modification of the procedure described by Medgyesi et

al (5). IgM was obtained by euglobulin precipitation by dialysis against 0.5% boric acid. The dissolved precipitate was then applied for gelfiltration on a Sephacryl S-300 column (Pharmacia Fine Chemicals). The peak obtained just after the void volume contained mainly IgM.

It has been claimed to be feasible to purify rat IgM by affinity chromatography on Con-A-Sepharose (24). I have been unable to reproduce this procedure, as most of the IgM appeared in the effluent and no IgM could be detected in material eluted with alpha-methyl-mannoside. The reason for this discrepancy has not been clarified, but may depend on a strain difference between the rats used. The use of Con A affinity chromatography for fractionation of rat immunoglobulins has not been reported from any other laboratory.

The effluent from SpA-Sepharose was precipitated with 40% ammonium sulphate and the precipitate was dialyzed against 5mM sodium phosphate pH 8.00. This Ig-fraction was applied to a DEAE-cellulose-column. After the column had been washed with 50mM phosphate buffer, the IgA-containing fraction was eluted by 0.3M phosphate.

The IgM- and IgA-fractions obtained by these procedures contained some detectable amount of IgG, mainly IgG2a and IgG2b.

6. Bacteriosorbents other than protein-A.

The purified rat IgG subclasses were radio-labeled with ^{125}I and used for screening of binding to bacteria

(paper I). Several species of Streptococci have been shown to carry Ig-binding structures analogous to the Protein-A of Staphylococcus aureus (10, 16, 25-30). The purpose of the work was to investigate the possible existence of distinctive rat subclass specificities within Ig-binding bacterial species with different Fc-receptor types. The definition of specificities different from that of Protein-A should increase the potential of using such affinity reagents as tools in the studies of rat immunoglobulins. Especially for IgG2a there is a need for an affinity matrix, as this subclass does not bind to Protein-A. Strains representing three different binding patterns for rat IgG were found: (1) Streptococcus equisimilis and human group G streptococci (FcR III), (2) Streptococcus dysgalactiae (FcR III or a new FcR type), (3) Streptococcus zooepidemicus (FcR V) (paper I). One human group G Streptococcal strain (G148) has been selected for further studies. It has been shown to be a true rat IgG adsorbent, i.e. it can quantitatively adsorb all rat IgG subclasses, without adsorption of IgM or IgA (Figure 2). G148-bacteria can also bind rat IgG complexed with antigen, even in the zone of antigen excess (Figure 3). The G148-bacteriosorbent can therefore be used as second reagent in radio immuno assays and for immunoprecipitation and isolation of antigens by the methodology described by Kessler for the use of S.aureus (31). The binding of all IgG-subclasses with varying affinity to G148 indicates its potential use in chromatographic fractionation. Even if the production of affinity matrices utilizing whole

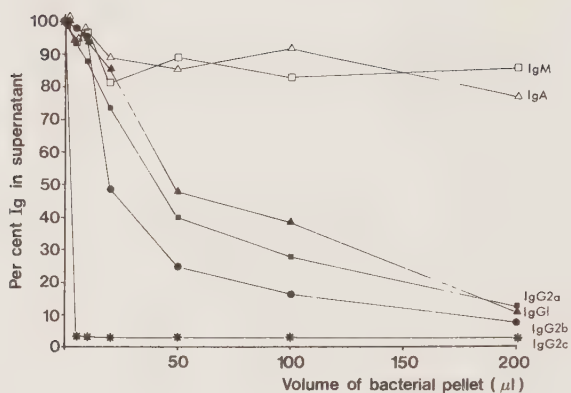


Figure 2 :

Absorption of rat serum immunoglobulins by G148 Human group G Streptococci. Varying volumes of bacterial pellets were suspended in 200 µl rat serum diluted 1/10 and were incubated for 1 h at room temperature. After centrifugation, the concentration of different rat Ig(sub)classes in the supernatant was determined by use of a radio immunoassay. Immunoglobulin concentration is expressed as percentage of unabsorbed rat serum.

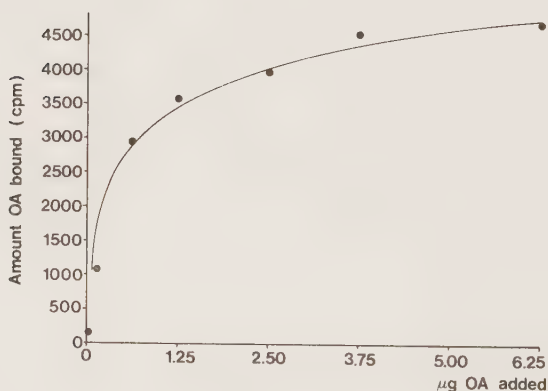


Figure 3 :

Absorption of immune complexes by G148 Streptococci. A constant amount of rat anti-ovalbumin (OA)serum (100 µl 1/200) was incubated with an increasing amount of radiolabeled OA for 2h at room temperature. Hundred µl 10% suspension of G148 bacteria were added and incubated for 1h at room temperature. The bacteria were washed and the radioactivity bound was determined.

bacterial cells has been reported (32, 33), the purified IgG-binding structure covalently linked to a chromatographic matrix (e.g. Sepharose) will be needed for efficient affinity chromatography.

Methods for solubilization and purification of the IgG-binding structure from G148, designated Protein-G, have recently been developed (34). Preliminary experiments with ^{125}I -labeled Protein-G have shown binding to all rat IgG subclasses and no binding to IgM and IgA. The binding specificity of soluble and Sepharose-bound Protein-G has to be further evaluated, as the specificity of bacteriosorbents may depend on the matrices used (paper II). The chromatographical quality of Protein-G Sepharose will also be analyzed when Protein-G is available in larger quantities.

Other bacterial species will be screened in order to find and develop bacteriosorbents with specificity for rat IgM and IgA, respectively.

PRODUCTION OF MONOSPECIFIC ANTISERA AGAINST RAT Ig (SUB)CLASSES.

1. Selection of animal species for immunization.

Sheep, goat, rabbit, and guinea-pig are the most widely used species for production of antiserum. My requirements were that the animals should be easy to handle and should produce an amount of serum feasible to use for both analytical and preparative purposes. Another important requirement was that the resulting immune sera must have high affinity for protein-A, as this will much simplify the preparation of IgG-fractions and the quantitative detection of bound IgG in various immuno assays. As only rabbits will fulfil these requirements, they were chosen for the immunizations. Two rabbits were used for immunization per immunoglobulin type, as there may be a large individual variation in the abilities of the rabbits to produce antibodies of good quality. The immunization schedule has been reported in paper III. Each rabbit produced about 200-300 ml of antiserum during a period of 1 to 1.5 year.

2. Principles of immunization.

Immunoglobulin molecules display a number of different immunogenic determinants. Some of these determinants are common to all classes of Ig, e.g. those present on the light chains, while others are class specific. Only very few determinants (1 - 4 amino acids) differ between the subclasses of IgG (37). The degree of immunogenicity of a particular immunoglobulin depends on the degree of

similarity (cross reactivity) between this immunoglobulin and the immunoglobulins of the species used for immunization. Various tactics have been used to improve the result of immunization against IgG subclasses. In order to decrease the number of common determinants, fragments of the IgG have been prepared, as most of the subclass specific determinants are located on the Fc-parts. Another widely used method is to induce immune unresponsiveness against the unwanted subclasses by intravenous injection of a large amount of a mixture of these subclasses, followed by intramuscular injection of the right subclass mixed with an adjuvant a few days later (35). This technique will not be successful if the mixture of "unwanted" Ig is contaminated with the Ig of interest (as they mostly are), as this can then induce unresponsiveness also against this Ig.

Most of the reported subclass specific antisera have been obtained after immunization with isolated myeloma immunoglobulins (3, 35). However, there may be a risk to use such monoclonal immunoglobulins as they may not be totally intact or they may be modified compared to normal immunoglobulins. The (sub)class populations display some degree of heterogeneity. Therefore, selection of one monoclonal Ig representing the whole population may result in antisera with varying reactivity against immunoglobulins within the same (sub)class. This has been reported for anti-IgE after immunization with monoclonal rat IgE (36).

To obtain antisera against rat IgG subclasses rabbits were immunized with preparations from normal serum. The use of such preparations was judged to be preferred compared to the use of monoclonal or myeloma immunoglobulins, as the risk of obtaining anti-idiotypic reactivities is diminished and as it gives an equal reactivity against the whole subclass population. Intact immunoglobulins were used as the fragmentation methods may result in harmful denaturations decreasing the immunogenicity and appearance of determinants not present on intact immunoglobulins. Treatments for induction of unresponsiveness were not performed as it has often been shown to create problems and to require large amounts of highly purified Ig-preparations.

Antisera obtained after immunization with IgM and IgA purified from serum showed reactivity mainly with IgG or no reactivity at all. This may indicate that rat IgM and IgA are less immunogenic in rabbits than IgG. Similar results have been reported for human immunoglobulins, showing variation in immunogenicity in rabbits (35). Immunization with monoclonal rat IgM and IgA from hybridoma cultures at much higher concentrations and purity resulted in antisera reactive with the heavy chains of these classes. In all rabbits immunized reactivities against common determinants on light or heavy chains were also detected.

3. Absorption and specificity testing of the antisera.

Besides Ig(sub)class reactivity the rabbit antisera also showed reactivity against common determinants on heavy and light chains or against determinants shared by more than one subclass. These cross reactivities had to be eliminated before the antisera could be regarded as (sub)class specific. Two methods can be used for this purpose. One involves addition of the crossreacting Ig in order to block and precipitate the antibodies, and by the other the crossreacting antibodies are removed by affinity chromatography (i.e. immunosorption). The addition of Ig to the antisera will result in the presence of soluble immune complexes that will induce many problems in the subsequent immunoassays, e.g. increased background in amplified immunoassays, nonspecific precipitates in gel diffusion techniques etc. Furthermore, it is not efficient to inhibit cross reactive antibodies of low avidity. Antisera made specific by this technique are not feasible to use for production of Ig(sub)class specific antibody-immunosorbents, as the specificity will be lost during elution of the columns.

Therefore, I have chosen to eliminate the cross-reactivities by immunosorption on purified Ig(sub)classes covalently linked to Sepharose. This will efficiently remove cross-reactive antibodies of low avidity and will result in antisera feasible to use both in amplified immunoassays and for the preparation of anti-Ig-immunosorbents.

In a first set of experiments, absorptions were made in order to obtain antisera showing monospecificity for the Ig(sub)classes as judged by the use of gel-precipitation techniques. Repeated absorptions on Sepharose columns with covalently linked Ig(sub)classes purified from normal rat serum had to be performed, before monospecific reactions were obtained in double immunodiffusion and immuno-electrophoresis.

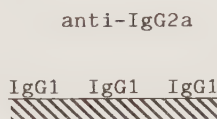
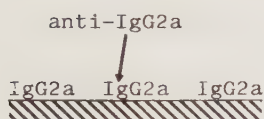
The specificity analysis of antisera is usually not carried further, even if the antisera later are used in amplified immunoassays. The sensitivity of amplified immunoassays is generally 1000 to 10 000 times higher than the sensitivity of gel-precipitation techniques, and the specificity must of course be analyzed with at least the same degree of sensitivity as the one later used in the assays. However, this often places the analysis in a vicious circle, i.e. for the performance of the test you need preparations of purified immunoglobulins in which the contaminating Ig is below the detection limit of the assay and to obtain these preparations you need antisera monospecific at this detection level. Without highly purified Ig-fractions it is not possible to differentiate between true cross-reactivity of an antiserum and contamination of the target antigen preparation (Figure 4). These problems have also been described for studies on monoclonal antibodies against human IgG subclasses (37, 38).

For the analysis of rabbit antisera against rat Ig(sub)classes we have developed a sensitive indirect

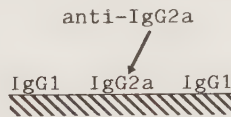
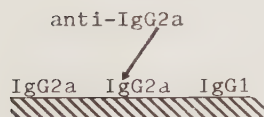
IgG2a - test target

IgG1 - test target

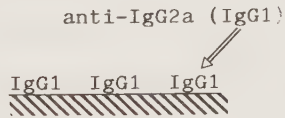
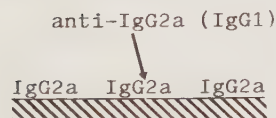
A: The ideal situation



B: Contaminated target Ig



C: Cross-reactive antibody



D: The typical situation

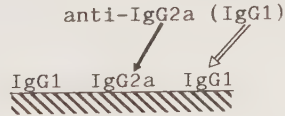
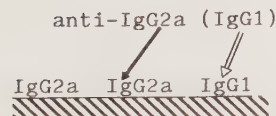


Figure 4 :

The great problem of specificity analysis of an antiserum. A detected reactivity against a control target antigen (e.g. IgG1) may be dependent on a true cross-reactivity of the antiserum (e.g. antibodies against IgG1 in the anti-IgG2a serum) or may depend on contamination of the control target antigen (e.g. presence of IgG2a in the IgG1 preparation).

^{125}I -labeled protein-A binding assay (III) using target preparations of monoclonal rat Ig adsorbed to the bottom of the wells of microtest plates. Monoclonal antibodies of the various isotypes were produced for this purpose by the hybridoma technology. After repeated cloning procedures these hybridoma-derived monoclonal immunoglobulins will have the advantage of purity by definition. The Ig(sub)class of the produced monoclonal immunoglobulins was determined by double immunodiffusion. The monoclonal Ig must be obtained from tissue culture to avoid the contaminating immunoglobulins present in ascitic fluid of animals with growing hybridomas. When testing the specificity in this sensitive assay, cross-reactivities were detected which could not be detected in double immunodiffusion. Additional absorption had to be performed before the antisera showed monospecificity at concentrations of solid-phase immunoglobulins at least ten times higher than those detected in the applied radio-immunoassays. These later absorptions were performed on columns with monoclonal immunoglobulins to avoid decrease in titer of the antisera, as will be the result if repeated absorptions are done with columns contaminated with the Ig of interest. Analyses of the specificity of antisera against IgM and IgA were performed with solid-phase monoclonal immunoglobulins obtained from hybridomas different from those used for immunization so as to avoid testing for reactivities against idiotypic determinants. By this methodology antisera against Ig(sub)classes have been produced, which have

demonstrated monospecificity in a sensitive radio-immunoassay (III).

RADIOIMMUNOASSAY FOR RAT IMMUNOGLOBULINS BINDING TO CELL SURFACE ANTIGENS.

1. Introduction.

Neoplastic cells may express structures, which distinguish them from their normal counterparts. These molecules may be located in different compartments, i.e. the cell membrane, the cytoplasm, or the nucleus. For the tumor immunologists the cell surface antigens are of particular interest, as these molecules are most exposed to the immune system. They may not only induce immune reactions against the tumor cells, but most importantly they may be the target for those immunological effector mechanisms which lead to tumor cell elimination. For a better understanding of the role of antibodies in the immune response against tumors, it is important to develop techniques making it feasible to analyze qualitatively and quantitatively the presence of antibodies of various Ig(sub)classes reactive against the tumor cell surface. Besides being a tool for researchers, such techniques may have practical applications for the immunodiagnosis and prognosis of cancer.

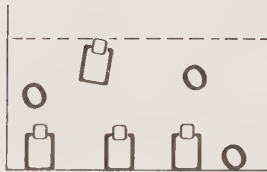
An important guiding principle during the establishment of the assay was that the method should be easy to make available for the clinical laboratory at the time when the human application is to be carried out. The performance should be simple, without need for sophisticated technologies or complicated equipment, and

it should be feasible to run large numbers of samples at high precision. The analysis utilizes the standard 96-well (8x12) microtest system. By use of this system the handling of large numbers of samples is simplified and devices for automatic dilution and pipetting of samples, washing of plates, spectrophotometric measurements, etc. are commercially available. Even without use of these devices, it is possible for one person to perform more than 1000 analyses per day.

The test established is a three layer radio immunoassay, in which rat antibodies bound to solid-phase antigens are detected by monospecific rabbit antisera against rat Ig(sub)classes (figure 5). The bound rabbit IgG is subsequently quantitated by the binding of radiolabeled Protein-A. This assay will be designated "binding test", as it does not measure any functional effects of antibodies but only the binding to test antigens.

2. Target antigen for the binding.

The source and properties of the target antigen will vary depending on the antibody reactivity studied. For analysis of antibodies against cell surface antigens, viable or fixed intact cells or cell membrane molecules, purified in various ways, can be used as target antigens. During the establishment of the binding test, alloantisera against the major histocompatibility antigens (transplantation antigens) of the rat (RT-1) were used for evaluation of the test properties. For this purpose living adherent monolayers of rat colon carcinoma cell lines grown in microtest plates were used



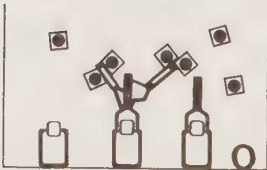
Target antigen in soluble form is adsorbed to the plastic wells or target cells are attached to the wells.



After washing, the wells are incubated with the rat serum to be analyzed.



After washing, the wells are incubated with rabbit antiserum against rat immunoglobulins.



After washing, the wells are incubated with ^{125}I -labeled Protein-A.



After washing, the radioactivity of the wells is measured.

Figure 5 :

The three layer radioimmunoassay (bindingtest), in which rat antibodies bound to solid-phase antigens are detected by monospecific rabbit antiserum against rat Ig(sub)classes. The bound rabbit IgG is quantitated by the binding of radiolabeled Protein-A.

as target antigen (III). The use of living cells as targets will not fulfil the criteria discussed above, however, as it requires laborious tissue culture procedures. The use of tissue cultured cells may also induce some other problems (V). The antigen must not detach from the solid matrix during the test and must be in large excess compared to the amount of antibodies in the test serum. The target antigen preparations must not contain any larger amount of rat immunoglobulin as this will increase the background. The establishment and evaluation of the binding test are presented in papers III and IV.

3. The first layer: the rat serum.

An advantage of this binding test is that the binding of various Ig(sub)classes can be evaluated without their purification or other pretreatments of the test sera. The untreated rat serum is diluted and directly added to the test wells. The binding has been shown to be linear over a wide range of concentrations. Therefore serum can be tested at a single dilution without any loss of information. It is of importance to control that the antigen is in large excess in order to avoid competition between different Ig(sub)classes. The optimal incubation time and temperature have to be analyzed for every antigenic system studied. For the studies presented in this thesis, incubation for 45 minutes at 37°C (cellular targets) or for 1 h at room temperature (protein targets) have been found to be optimal. However, in studies of cell membrane proteins solubilized by detergent we found incubation over night at 4°C to be

preferable (39).

4. The second layer: the rabbit antisera.

The isotypes of the bound rat antibodies were analyzed by use of the six rabbit antisera against rat Ig(sub)classes. As the bound rabbit IgG will be quantitated with Protein-A in a subsequent step, no preparation of Ig-fractions for labeling was required. Since the purified Ig-fractions would be more sensitive to denaturation and aggregation, their use should increase the background and should decrease the reproducibility of the assay.

The sensitivity of the binding test will be dependent on the portion of the rabbit antibodies that will bind Protein-A and the competition between these antibodies and non-binding antibodies. Maximal sensitivity would be obtained if all the bound rabbit antibodies had affinity for Protein-A. If all rabbit antibodies are not protein-A binding, the sensitivity may be increased by the use of IgG-fractions of the antisera isolated by affinity chromatography on Protein-A-Sepharose. This has been evaluated for all the six rabbit antisera used, by comparisons of the maximal binding obtained by unseparated antisera and Protein-A purified IgG-fractions. Since no differences could be detected, it may be concluded that the majority of the antibodies in these antisera are Protein-A binding and that no advantage would be gained by the use of isolated Protein-A-binding fractions.

5. The third layer: labeled Protein-A.

It is a great advantage to use radio labeled Protein-A, since it allows labeling to high specific activity, is available at high purity, and has high affinity for rabbit IgG (15, 17). The use of labeled Protein-A instead of labeled anti-Ig increases the sensitivity of the test as the additional layer will result in an amplification of the response and the background binding of radiolabeled Protein-A is often low compared to radiolabeled anti-Ig (40, 41). It is also easier to keep one reagent radiolabeled than six different reagents, which in addition would require Ig fractionation before labeling. The low degree of Protein-A binding directly to rat immunoglobulins simplifies the (sub)class analysis as the background is low for (sub)classes not detected by the intermediate step of rabbit antiserum, i.e. the "spill-over effect" is very low.

In the initial studies we used Protein-A labeled with carrier-free ^{125}I by use of the lactoperoxidase method (42, III). We have later used Protein-A labeled with ^{125}I by use of the Bolter-Hunter method (43, IV - VI), as this results in higher specific activity and the ^{125}I -SpA can be successfully used after storage for much longer periods of time due to less denaturation and less increase of the background binding. Protein-A may also be labeled with several other tracers including other isotopes, enzymes, fluorescent probes, metal ions etc. (41). We have also adapted the binding test to an enzyme-linked immunosorbent assay (ELISA), utilizing

horseradish peroxidase conjugated Protein-A (obtained commercially from Sigma). However, we have found , in accordance with others (50), the radioimmunoassay to have higher sensitivity than the ELISA.

6. Qualitative and quantitative aspects of the assay.

The specificity of the test was shown by criss-cross studies of alloantisera tested on both syngeneic and allogeneic target cells. No positive reaction was detected against the syngeneic cells, even if the reaction was strong against allogeneic target cells. This also showed the feasibility of simultaneous analysis of antigen binding antibodies of different (sub)classes. Even at high concentrations of these positive sera no competition between different Ig(sub)classes could be demonstrated, indicating that the antigen was in large excess. The antibody binding was shown to be linear over a wide range of concentrations. One implication of this is that serum can be tested at a single dilution without any loss of information. By saturation experiments (a constant cell number was incubated with an increasing amount of rat antibodies, (III)), it was shown that the response is only dependent of the first layer in the test, the amount of rat Ig. Limiting second or third reagents would result in a decrease of the bound radioactivity at higher rat Ig concentrations (62).

The reproducibility of the binding test was analyzed by repeated testing of a panel of sera of different

quantitative and qualitative binding patterns. The day-to-day variation was shown to be 10% when living cells were used as targets and 5% for protein targets. The inter- and intra-plate variations were both shown to be less than 1% of the mean. This low intraplate variation indicates that the use of more than two parallels would not be of any advantage.

7. Non-specific binding of normal rat sera.

In order to differentiate between negative and positive responses in the binding test, the binding levels of normal sera have to be known. The binding of each individual serum was compared to the binding of a normal reference serum pool (NRS) and expressed as the binding ratio between test serum and NRS. A large number of sera from untreated rats of various ages, and of different sexes, strains, and nutrition statuses were collected to determine the antibody-binding levels in normal rats and for investigation of the frequency of falsely positive sera. The Ig-binding levels were shown to be normally distributed and to have homogeneous variances. The means and standard deviations were calculated and the number of falsely positive sera determined (III). When the reactivities of these falsely positive sera were further analyzed, it was shown that the majority bound directly to the plastic of the test wells (IV). These non-specific reactions were unusual and different from previously described background reactivities, since they were detected in a test system with precoated plates (20% fetal calf serum, FCS) and with test serum supplemented with a high protein concentration (20% FCS)

during incubation. Neither further coating with proteins nor addition of detergent was effective in eliminating the reactions. The binding varied with the type of plate used, and the binding did not reflect a true immunochemical interaction with molecules adsorbed to the plastic surface (FCS or rat serum proteins).

Two different approaches were established to eliminate the non-specific reactions. When living cells are used as target antigen in the binding test, exclusively cell-bound radio-activity is eluted with the non-ionic detergent Nonidet P40, which solubilizes the cell membrane without breaking the non-specific interactions. When rat antibodies against soluble antigens adsorbed in the test wells are analyzed, a plate is used that does not show this non-specific binding or for which the reactivity can be avoided by inclusion of 0,05% Tween 20 in the incubation mixture. By these methods most of the non-specific reactivity is avoided and the frequency of falsely positive sera is only two per cent or lower (IV).

The described non-specific binding to plastic may be under genetic influence, as the two strains of rats studied display very different distributions of the plastic-binding Ig of the various Ig(sub)classes. IgG2a and IgG2b dominate in BN rat sera, while binding of IgA and IgM are most frequently observed in the WF-strain. In the F₁-hybrids of BN male and WF female (WB) the frequency of plastic-binding Ig is not restricted to any particular Ig(sub)class (table 2 and figure 6).

Table 2. Percentage of false positives in sera from untreated rats.

Limit ¹⁾	Strain ²⁾	IgG1	IgG2a	IgG2b	IgG2c	IgM	IgA
2,0	WF	1,5	4,5	7,6	12,3	17,9	20,9
	BN	9,8	25,0	30,6	8,3	6,6	0
	both	5,5	14,2	18,8	10,1	12,6	11,0
2,5	both	1,6	8,7	8,6	1,6	6,3	5,5

- 1) Mean binding ratio + S.D. (=limit 2.0) and mean binding ratio + 2 S.D. (limit 2.5) are used to define positive sera.
- 2) Sera of 65 - 67 WF and 60 - 62 BN rats were assayed for binding of each of the indicated immunoglobulins.

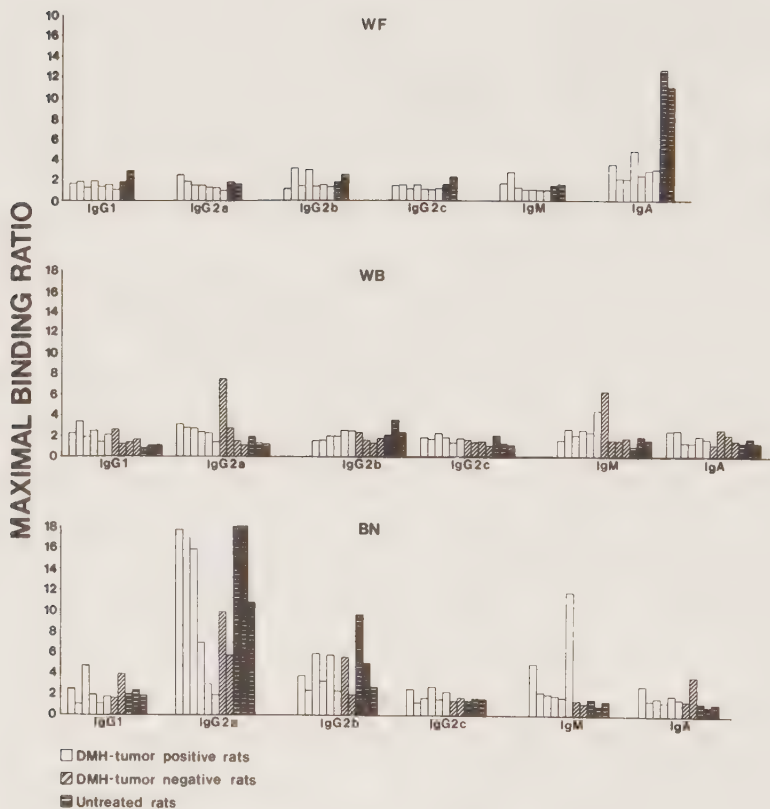


Figure 6 :

The non-specific binding detected in sequential sera from WF-, BN-, and WB-rats, untreated or treated with DMH. The maximal Ig-binding ratio found for each rat, when tested on FCS-coated plates of tissue-culture grade, is presented. SDS (1%) was used for elution of bound reactivity. The rats were followed for 1-1 1/2 years.

ANALYSIS OF ANTIBODIES REACTIVE AGAINST CHEMICALLY
INDUCED RAT COLON CARCINOMA.

1. Previous serological studies of rat colon carcinoma:

Chemically induced rat colon carcinomas have been used extensively at the department of Tumor Immunology, the Wallenberg Laboratory as an experimental model for studies of the immune responses against tumors. Rats are given repeated subcutaneous injections of 1,2-dimethylhydrazine and after a latency period of 6 - 12 months tumors will appear in the colon (53). In vivo and in vitro studies have shown that these tumors may present individually distinct transplantation antigens, common-colon carcinoma associated antigens, and antigens cross-reactive with embryonic tissues (44-49). The serological studies have mainly involved tests of immunological effector activities, analyzed by cell biological methods and no molecular characterizations have been performed. Most of the studies have been concentrated on analysis of the "serum blocking factors" (SBF) that inhibit the specific lymphocyte cytotoxicity against tumor cells (54). Immune complexes between tumor antigen and tumor reactive antibodies have been claimed to be one type of such blocking factors (50). They have affinity for Staphylococcus aureus bacteria (51) and bind to immunosorbents of antiserum against rat IgG (52). These results suggest that the antibodies involved in the serum blocking activity belong to the IgG1 or IgG2c subclasses. However, methods for evaluation of the Ig(sub)class content of the immunoglobulins bound to the

S. aureus bacteria and the anti-IgG-column were not available at that time.

Analyses of sera active in complement mediated cytotoxicity have been performed by microcytotoxicity assay techniques (51), in which the cells remaining after the cytotoxic reaction are stained with crystal violet and counted under the microscope, and by ⁵¹Chromium release techniques (55), in which cell damage is measured as release of ⁵¹Cr-isotope during the test. Complement sources used have been either rabbit or rat untreated sera from normal animals. The lack of affinity for S. aureus bacteria and the activity with rat complement (51) may indicate that these antibodies are of the IgG2a and / or IgG2b isotypes, as rat IgM does not function with rat complement (7). This is not in accordance with the experiments performed with an anti-rat IgG column (52), as the complement cytotoxicity mediating antibodies were found in the effluent of the column. However, the specificity of the immunosorbent procedure was not clarified with regard to individual Ig(sub)classes. In a later study (49) rat IgM was shown to mediate both complement cytotoxicity with rabbit complement and antibody-dependent-cellular cytotoxicity (ADCC) together with Sephadex G10-passed spleen cells from a normal rat. However, again the specificity for individual Ig(sub)classes of the immunosorbent used is not known.

Antibodies in sera of colon-carcinoma bearing rats have also been detected by an indirect immunofluorescence

binding assay (57). Embryonic tissue was homogenized and covalently linked to agarose-beads, while thymic tissue was used for similar production of control beads. After the incubation with rat test sera, the beads were washed and incubated with fluorescein-labeled anti-rat IgG. After another wash, the percentage of stained and unstained beads was determined by counting of 100 to 200 beads under the microscope. By this test antibodies against embryonic antigens could be detected in sera from multiparous rats and from colon carcinoma bearing rats with titers of 1/10 000 and 1/100, respectively. Only two sera out of 130 displayed non-specific binding to the thymic-antigen beads. The assay was only performed with an anti-IgG reagent and the reactivity of this antiserum to individual Ig(sub)classes cannot be evaluated. The test is rather laborious involving counting of beads under the microscope. It has not been possible to change to a radiolabeled reagent instead of the fluorescein-conjugated, mostly because of background problems (Å. Boketoft, personal communication). Tumor tissues have not been tested as targets in this assay.

The specificity of the serological reactions described above has mainly been evaluated by two-target analyses. The reactivities or binding of sera have simultaneously been tested against a colon-carcinoma target and a control target, either non-crossreacting tumor cells or normal cells. However, in analysis of the cross-reactivity between colon carcinoma and embryonic tissues, in which absorptions of multiparous sera were

performed with intact cells from tumor-, fetal-, and normal tissues, the material bound to the cells during absorption was also eluted and analyzed (45).

2. Target antigen for testing of tumor-binding Ig:

Initially the described indirect ^{125}I -SpA binding test for detection of antibodies against rat colon carcinoma was directly applied using viable attached tissue cultured colon carcinoma cells as target antigen. No cell-binding serum could be detected among the 400-500 sera tested. The reasons for this was not further evaluated. Although a number of different cell lines were tested, it may have depended on selection of weakly antigenic cell-variants during explantation and growth of the tumor cells in vitro. This would lead to a low density of antigens in the wells at monolayer growth and consequently to a low sensitivity. Examples of the successful use of intact cells as targets in similar binding tests have been reported by others for chemically induced rat fibrosarcomas (58), chemically induced rat sarcomas (59, 60), and for tumors in other species.

In the rat colon carcinoma system it was necessary to evaluate the use of non-cellular targets in the binding test, i.e. to find a suitable fractionation method for cell membrane antigens without the support of an existing cell target assay. The various antigen preparations therefore had to be tested against a number of different, randomly selected sera from rats which could be anticipated to have antibodies reactive with

colon carcinoma tissues. Cell surface molecules may be solubilized from whole cells or from purified plasma membranes by a variety of technique. The different extraction procedures used in this study will be discussed in the section about studies on tumor associated antigens.

The extracts were tested in the binding test after adsorption to the bottom of plastic microtest wells. This may constitute a problem as the adsorption capacity of the plastic is limited to about 0.2-1.0 μg protein / well (61). When crude material is used the protein of interest is only a minor part of the total protein content. Due to the limited number of binding sites and the different affinities of proteins for these sites the protein of interest may be adsorbed in very small quantities due to competition with the other proteins (62). This will result in a low sensitivity of the assay. The influence of the purity of the solid phase antigen on the sensitivity have clearly been demonstrated in an enzyme immuno assay for human IgG (63).

Antigenic material could be detected in one of six different extracts tested. This extract was obtained by the single-phase 1-butanol extraction procedure described by LeGrue et al (64) for preparation of tumor specific transplantation antigens from chemically induced murine fibrosarcomas. These authors have not performed any serological analysis of these extracts,

but only in vivo immunobiological studies by immuno protection assays. At the time of the initial work on the butanol extracts (V), a serological analysis had been reported using butanol extracts of human sarcomas fixed to the wells by glutaraldehyde (65).

The extracts obtained by this 1-butanol extraction procedure are designated crude butanol extracts (CBE), and the included antigens detected with colon carcinoma immune rat sera are designated butanol extractable tumor antigens (BETA). The BETA are not very sensitive to competition with non-BETA-proteins and the CBE can be tested over a wide range of concentrations without any decrease in binding even at high concentrations of the extracts. The pH and ion strength do not dramatically influence the adsorption indicating that the BETA bind to the wells by strong hydrophobic interactions. The adsorption is also possible in the presence of 1-butanol. CBE have been stored at -80°C for more the one year without decrease in antigenicity.

The use of CBE provides a continuous and consistent source of antigen for detection of colon carcinoma binding antibodies. The reproducibility of the assay has been shown to be high with a day-to-day variation of less than five per cent. The use of CBE may also increase the sensitivity of the assay of antibodies with demonstratable binding to intact cells. As an example a hybridoma supernatant containing syngeneic monoclonal antibodies binding to a spontaneous kidney carcinoma displayed a binding of about 1000 cpm to a monolayer of

intact cells, while a binding of about 16 000 cpm was obtained against a butanol extract of this tumor containing the reactive antigen (Magnus Lindvall, personal communication).

3. Specificity analysis of a demonstrated antibody binding:

The first level of specificity analysis is a two-target analysis performed by parallel testing of the sera on test and control target antigens. This will exclude sera reacting with molecules present in both targets and sera with background binding to the plastic. It might be difficult to evaluate the result if the targets show very varying background reactivities. An important possible risk is that the antigens may be present in both preparations, but are competed out by molecules in one of the preparations during adsorption to the wells. However, if a serum reacts with two targets, other methods are required to analyze if this serum contains one population of antibodies reacting with the same molecules on the two targets or if it contains two independent populations of antibodies reacting with different molecules on the two targets. For distinction between these alternatives, the binding test has to be combined with adsorption or inhibition studies with the different antigens.

Two advanced methods for specificity testing of reactivity against CBE have been established, the column

immunosorption technique and the competitive binding inhibition assay (V). For immunosorption, crude butanol extracts were covalently linked to activated CH-Sepharose 4B. A rather small amount of protein (0.5-1.0 mg) was coupled per ml Sepharose to ensure that the majority of the protein will be coupled to the matrix. Two variants of solid phase adsorption are possible, the batch technique and the column procedure. The use of columns is more efficient, and the chromatographic process will make it useful also for adsorption of antibodies with relatively low avidity. By the same volume of sera required for direct testing in the binding-test, it is possible by this method to absorb the reactive antibodies effectively with the appropriate extracts. However, this method provides mainly qualitative information about the binding specificities of the sera.

For quantitative analysis of specificity, a competitive binding inhibition assay was used. The soluble antigens were titrated for their ability to inhibit the binding of simultaneously added antibodies to antigens adsorbed to the wells. After incubation, the mixture was aspirated and bound rat Ig quantitated in the binding test. This method can also be used to quantitate the relative antigenicity of various antigen preparations and the degree of cross-reactivity between different tumor extracts and to detect the BETA during various fractionation procedures.

These two methods might also be used for specificity

analysis utilizing intact cells. If the antigens studied are not sensitive to enzyme treatment, single cell suspensions can be used in the competitive binding inhibition assay. For solid-phase immunosorption, columns with cells grown on a micro carrier matrix (e.g. cytodex) may be used.

With the two established techniques for specificity analysis, tumor-reactive antibodies detected in sera of rats immunized with tumor isografts were shown to be directed to tumor associated butanol extractable antigens shared by some DMH-induced rat colon carcinomas but not expressed by normal tissues.

An additional method involving immunohistological technique (88) for specificity analysis on the tissue level is of great importance. Such a technique is under investigation (a cooperation with Thomas Brodin) using Ig-fractions of reactive sera or immunoaffinity purified antibodies labeled with biotin and incubated on thin layer tissue sections obtained from various in vivo tissues. The bound antibodies will be detected by Horseradish peroxidase-conjugated Avidin and diaminobenzidine substrate. By this method it should be possible to study the binding to different cell types in a tissue and also to get information on the cellular localization of the antigens, i.e. whether they are present on cell membranes or are located in the cytoplasm or the nucleus.

Finally, an analysis of the molecular specificity is being performed in cooperation with Bo Jansson aiming at an analysis of "finger prints" characteristic of the studied serum. The crude extracts are separated by electrophoresis, i.e. polyacrylamide gel electrophoresis or isoelectric focusing, followed by a transfer (blotting) to nitrocellulose (74). The antigenic bands are detected by incubation with reactive rat sera, followed by rabbit anti-Ig and horseradish-conjugated Protein-A and finally diamino benzidine substrate (75). This will result in a number of bands (fingerprints) characteristic for the analyzed serum. By this method it may be possible to compare the reactivity pattern of various sera against different antigenic molecules present in the same extract. It might also be possible to immunoprecipitate radio labeled extracts with rat serum and detect the molecules after electrophoresis of the precipitate by autoradiography (31).

These two methods, the immunohistology and the electrophoretic analysis, function today with some monoclonal antibodies against butanol extracts, but must be further optimized before analysis of serum antibodies will be feasible.

STUDIES ON TUMOR-ASSOCIATED ANTIGENS.

1. Introduction and previous studies on colon-carcinoma associated antigens.

Tumor antigens are defined as molecules that can elicit an immune response in the host and/or react with induced immune mediators, i.e immune lymphocytes or antibodies. The tumor antigens may reside on the cell surface or be localized intra-cellularly. The antigens may induce an immune response leading to rejection of the tumor, or they may induce immunosuppression resulting in facilitated growth of the tumor. Some tumor antigens will induce antibody production in the host, resulting in the various reactivities discussed above. The tumor antigens may not always appear in direct association with the malignant transformation, but may sometimes be a secondary effect of the malignant growth. The detection of tumor antigens and immune responses against them is very dependent on the technology used for the analysis, and the time when the tests are performed relative to tumor development. Therefore it is not meaningful to speculate about how frequent and important immune responses against tumor antigens are in general in the malignant diseases on the basis of one type of test performed on one occasion.

There can be several reasons why a tumor antigen can induce an immune response in the autochthonous host (66):

1. Alteration of a normal molecule as a result of changes in the tumor cell physiology can produce a new antigenic determinant. For example changes in glycosyl transferases result in altered sugar moieties in glycoproteins or glycolipids.
2. Reexpression of fetal- or differentiation related proteins dependent on disturbances in the regulation of gene activity.
3. Increased concentrations of normal cellular proteins in transformed cells.
4. Complexes between normal proteins and tumor protein may induce immune responses against both the normal and the new protein by a carrier-hapten like effect.
5. The tumor cells may contain new or rearranged genetic information resulting in new molecules.
6. Expression of viral or virus associated proteins after transformation.

This list of possibilities illustrates that the tumor antigens are very diverse and no general properties of the antigens can be described. Knowledge about the nature of tumor antigens in one tumor system cannot be directly applied in other systems. However, these discrepancies between various tumor antigens may not always be of importance in the use of the respective tumor antigens for immunotherapy and diagnosis of tumors.

It has been shown that the rat colon carcinoma cells display individually unique tumor antigens, common colon carcinoma associated antigens, and antigens cross-

reactive with embryonic tissues (44-49). The oncofetal antigens were found to be of two types, one being fetal colon specific and the other being more wide-spread among various embryonic tissues (45). Cross-reactivities have been demonstrated for colon carcinomas induced by three different carcinogens, N-methyl-N-nitro-N-nitrosoguanidine, 1,2-dimethylhydrazine and 3,2-dimethyl-4-aminobiphenyl. Therefore the antigenicity is not dependent on the type of carcinogen used for induction (44). These different classes of tumor antigens associated with colon carcinoma have been studied in vivo by criss-cross immunizations analyzed with immunoprotection assays (48) and immunoprevention experiments, in which primary tumors are induced by carcinogen in animals immunized against colon carcinoma or control tumors (67-69). The embryonic antigens have also been studied by analysis of the immunity in multiparous rats against colon carcinoma (45,48) and tumor induction in multiparous rats (68,69). Immunization with fetal tissues may also induce protection against challenge with colon carcinoma cells (48,70,71). Immunotherapy has been performed after surgical removal of primary colon carcinomas by immunization with irradiated colon carcinoma cells (72).

These different types of tumor antigens have also been studied by in vitro techniques. The common colon carcinoma associated antigens have been detected by microcytotoxicity assays of lymphocyte reactivity (44). Serum blocking activity displayed the same type of specificity (45,54). Serological studies of antibody

reactivity (by complement cytotoxicity, ADCC and binding tests) are in accordance with these results (49,57).

Soluble fractions of a colon carcinoma, containing tumor associated and embryonic antigens, have also been studied (73). The antigens were detected by their ability to inhibit the cytotoxicity of lymph node cells, obtained from animals immunized against colon carcinoma, against tumor- and embryonic target cells. Lymphocytes from multiparous rats were used for detection of embryonic antigens. The animals were immunized with a colon carcinoma different from the one used as antigen source. Therefore cross-reacting and embryonic antigens were analyzed, but not the unique antigens. Cell membrane antigens were solubilized by papain treatment or 3 M KCl treatment of homogenized tumor tissue. Antigenic reactivities could be found in both the high and low molecular weight fractions obtained by Sephadex G-200 gel filtration of 3 M KCl solubilized tumor material. These fractions, containing about 70 % of the material applied, displayed both cross-reactive and embryonic reactivities. DEAE-ion exchange chromatography of papain solubilized cell membrane proteins also indicated a great heterogeneity of the tumor antigens. One fraction was eluted early and contained cross-reactive and fetal colon specific antigens, another fraction showed strong binding to the DEAE-column and contained mainly fetal colon specific and fetal widespread antigens (73). Even by the low resolution obtained in these studies, it was indicated that the

three antigenic reactivities, i.e common colon carcinoma antigens, fetal colon specific and fetal wide-spread antigens, might be feasible to separate by chromatographic procedures. The difference between fetal colon specific and fetal wide-spread antigens was further confirmed by affinity fractionation by multiparous sera (73) and cell absorption studies on multiparous sera (45).

A different method for analysis of cell mediated immunity against colon carcinoma, a leukocyte adherence inhibition assay (LAI), has recently been described (76,77). By this method, immune reactivity is measured as leukocyte detachment from a solid surface following stimulation with soluble antigens. Tumor tissue homogenized in phosphate buffered saline (PBS) is used as the antigen source. It may be feasible to use the LAI-assay also for detection of antigens in various fractionated tumor extracts. Similar PBS-homogenates of embryonic tissue have been used as targets for tumor associated antibodies in a binding test (57).

Antigens detected by assays for cell-mediated immunity or by immunoprotection assays are not necessarily identical to the antigens reactive with antibodies. Studies on a murine leukemia virus induced lymphocytic neoplasm (RBL-5) showed that the immunoprotective antigens could be separated from antigens reactive with syngeneic serum by gel filtration of detergent solubilized cell membranes (78).

2. Preparation of soluble antigens associated with rat colon-carcinoma.

A variety of techniques exists for solubilization of molecules from cell membranes. I have evaluated six different solubilization or extraction methods in order to obtain an antigen source feasible to use as target antigen in the binding test for tumor associated antibodies. These experiments included solubilization by 2.5 % 1-butanol (64), 3 M KCl (79), 0.2 % deoxycholate (80), 1 M Urea (81), 4 M Urea (82) and PBS-homogenization (76). All these extracts resulted in rather crude preparations, i.e numerous bands were detected after SDS polyacrylamide gel electrophoresis. The protein yield ranged from 4 to 40 mg protein per ml sedimented tissue suspensions. The extracts were dialyzed against PBS and adsorbed to the plastic wells for use as target antigens in the binding test. Reactive sera were only detected against the butanol-extracted material. Therefore this procedure was used in the continued studies. The antigenic material might also be present in some of the other extracts, but might not be detected by this method depending on competition with the bulk of non-antigenic molecules during the antigen adsorption step (see above).

The butanol extraction was performed as described by LeGrue et al (64), but the trypsinization of the tissues before extraction was excluded (V). The mechanism of butanol extraction is not known. It has been shown, however, that butanol distributes between the solution

and the membrane and increases the membrane fluidity (83). The extraction is non-cytolytic, which results in diminished contamination with cytoplasmic molecules or with molecules from intracellular membranes. In accordance with previous reports in other species (84), the rat major histocompatibility antigens (RT-1) are not extracted and therefore the extracts can be used also for analysis of allogeneic tumor immunizations (V). Beta-2-microglobulin has been claimed to be associated with tumor antigens of human colon carcinoma (85), but beta-2-microglobulin could not be detected in the butanol extracts of rat colon carcinoma. A normal cell protein, p53, has been shown to be associated with transformation specific proteins and antibodies against p53 are sometimes detected in sera of tumor-bearing animals (66). However, the p53 was not detectable in the butanol extracts of rat colon carcinoma. The possible presence of beta-2-microglobulin and p53 was evaluated by analysis of the binding of a rabbit anti-beta-2-microglobulin serum and a monoclonal antibody against p53 (PAb 122,(89)) to the adsorbed extracts.

The butanol extraction procedure was found to be a very simple and reproducible method to obtain material that can be used as target antigen in the binding test. The extraction resulted in about the same amount of protein and in about the same antigenicity every time it was performed. The CBE has been shown to contain cross-reactive colon carcinoma associated antigens. These antigens include several antigenic components which seem to be unequally distributed among the various colon

Table 3: Binding of colon-reactive sera to
various crude butanol extracts.

Tissue extracted	t u m o r i m m u n e s e r u m		
	WF-anti-W49-1	WF-anti-W49-2	BN-anti-W163
WF-colon carcinoma - W49	+	+	-
WF-colon carcinoma - W163	+	-	+
WF-colon carcinoma - W6038	+	+	-
BN-colon carcinoma - BN7014	-	-	+
WF-kidney carcinoma - Wk2027	-	-	-
WF-T-lymphoma - W62	-	-	-
WF-normal colon	-	-	-
WF-normal kidney	-	-	-
WF-normal liver	-	-	-

carcinomas (table 3). The BETA are not likely to be autoantigens of the colon as they are not present in extracts of normal colon and it has been shown to be difficult to induce autoimmunity against colon tissue in the WF-rats (86). The presence of embryonic antigens in the extracts of rat colon carcinoma has not been tested.

Besides being very useful for serological analyses, the crude butanol extracts also have the potential of being useful as an antigen source in the leukocyte adherence inhibition (LAI) assay (76,77, Inagaki et al, unpublished results).

3. Partial biochemical characterization of butanol-extractable tumor antigens (BETA).

The existence of tumor antigens in neoplastic tissue is one of the fundamental corner stones in tumor immunology. Surprisingly little is yet known about their physiochemical properties, and very few antigens have been purified and their molecular properties studied. The most detailed studies are reviewed by Law et al (78) and include five different experimental tumor models. Most attempts to isolate tumor antigens have been based on techniques developed for the isolation of histocompatibility antigens, even if the similarities between the two groups of antigens are unknown. One major problem in the analysis of tumor antigens has been to find reliable methods suitable for detection of the solubilized antigens and for assessment of the degree of purification achieved. Inadequate purity or specificity

of reagents and the lability of antigens have contributed to this problem. Despite considerable efforts to use syngeneic tumor immune sera in the assays, quantitation of soluble tumor antigens has remained elusive (87).

The crude butanol extracts (CBE) have been found to be very useful as a starting material for purification and characterization of the butanol extractable tumor antigens (BETA) present in colon carcinoma tissues. The BETA can be detected by use of the described binding test and can be quantitated by the competitive binding inhibition assay, utilizing syngeneic tumor immune sera shown to be specific for the BETA of rat colon carcinoma (V,VI). The adsorption of the BETA to the plastic wells is relatively independent of the pH or the ion strength of the buffer used, and a wide range of concentrations of CBE can be used. This makes the detection of BETA relatively easy, as no titrations or laborious buffer changes are needed before analysis of the fractions obtained after various chromatographic procedures. It will also make chromatography at a small analytical scale feasible, which will be faster and consumes less reagent. It should be stressed that this mode of analysis provides mainly qualitative or semi-quantitative results. For a real quantitation of BETA-concentrations the competitive binding inhibition assay has to be used.

The BETA have been characterized mainly by their behavior when applied to different chromatographic

procedures (VI). Tumor antigens may be proteins or glycolipids, and this will greatly influence the selection of methods for characterization. Protease treatments of adsorbed crude extracts with proteases with different specificities, trypsin and dispase, showed that BETA are proteins or are associated with protein.

Adsorptions of CBE on small columns with various lectins coupled to agarose indicated that the BETA do not bind and consequently do not include glycoproteins with any of the widely distributed sugar moieties.

Molecules extracted from cell membranes should be expected to be hydrophobic, and to have affinity for matrices for hydrophobic interaction chromatography. This was first studied by adsorption of CBE on small columns of phenyl- and octyl-Sepharose. The BETA had high affinity for both octyl- and phenyl-Sepharose even at physiological ion strength. The binding to phenyl-Sepharose was lower than to octyl-Sepharose, but could be increased by addition of 4 M NaCl to the buffer. Full-scale chromatography showed that the majority of the proteins in the crude extracts does not bind to the phenyl-Sepharose at 4M NaCl. The BETA bound, and could be eluted by a linear descending salt gradient ranging from 4 M to 0 M NaCl in 5 mM sodium phosphate buffer pH 7.3. This elution indicated a heterogeneity in hydrophobicity of the BETA, as they eluted in two well separated peaks. This separation may be further improved

by use of another gradient shape or stepwise elution. These experiments showed that the use of hydrophobic interaction chromatography may be effective in the purification of membrane molecules, comparable in resolution to many affinity chromatography methods.

Anion exchange chromatography was also found to be an effective procedure for purification of the BETA. At pH 6.0 most of the proteins (90 %) bound to the Mono-Q column (Pharmacia Fine Chemicals) and could be eluted by a linear NaCl gradient. The antigenic material was detected in fractions that include only about 10 % of the proteins. These fractions included eight detectable peaks on the chromatogram, but the connection between these peaks and the BETA is not known. Later chromatofocusing experiments have shown that the separation by ion exchange chromatography may be further improved by running the separation at pH 4.0 instead of pH 6.0, as only a minor fraction of the proteins, including the BETA, will bind to the column at pH 4.0.

In order to estimate the pI of the BETA, chromatofocusing experiments were performed utilizing the FPLC-system with the Mono-P polybuffer exchange column and the polybuffer 74 (Pharmacia Fine Chemicals). The BETA bound to the column at pH 6.3, but was not eluted by the pH-gradient ranging to pH 4.0, which is the low pH limit for this method. The antigens could be detected, however, in the NaCl eluate following the pH-gradient elution. This shows that BETA belong to a group of acid proteins with pI below 4.0, but the exact pI and

the heterogeneity according to pI cannot be established by this method. Immunoblotting and immunoprecipitation techniques in association with isoelectric focusing are being established to improve this analysis. These techniques also have to be used in connection with SDS-polyacrylamide gel electrophoresis for estimations of the molecular weight of the BETA.

These studies have shown that rat colon carcinoma associated antigens can be extracted from tumor tissues by a single-phase solution of 2.5 % 1-butanol. The antigens belong to a group of hydrophobic, acid proteins that do not contain any widely distributed sugar moiety. A combination of hydrophobic interaction chromatography on phenyl-Sepharose and ion exchange chromatography on Mono-Q anion exchanger at pH 4.0 will result in a high degree of purification. These data will function as a basis for further characterization of the antigens, and will be important in the continued studies of their biochemical and immunobiological properties. However, the possible existence of other types of rat colon carcinoma associated antigens not extractable by 1-butanol must be stressed and must not be forgotten in the continued studies of the molecular immunology of rat colon carcinoma.

SUMMARY AND FUTURE PROSPECTS

The immune responses against tumors are very complex and involve several different immune mechanisms and different immune mediators. For a better understanding of these immune reactions and their complex interactions, it is very important to be able to resolve the different mechanisms and perform detailed molecular and immunobiological studies on the reactants involved. Therefore, there is a great need for advanced technology that makes such studies feasible. The work summarized in this thesis has been concentrated mainly on the establishment of methods for analysis of tumor associated antibodies in serum and characterization of the antigens defined by these antibodies. The developed technology has been applied to studies on chemically induced rat colon carcinoma.

Rat Ig(sub)classes can be fractionated from serum by use of established ion exchange- and affinity-chromatography procedures at both analytical and preparative scale. The methods will be useful in the analysis of the tumor immunological functions of the various immunoglobulin types. Which Ig(sub)classes mediate cytotoxicity in connection with complement and/or ADCC-mediating leukocytes? Are the isotype important for the function of idiotypic antibodies (59)? What is the connection between the production of antibodies of various (sub)classes and rejection of tumors?

Rabbit antisera against six different rat Ig(sub)classes have been produced. The antisera have been shown to display monospecificity also at the sensitivity level of amplified immunoassays. These antisera are used for detection and purification of the various Ig(sub)classes.

A radio immunoassay for qualitative and quantitative antigen binding analysis of rat immunoglobulins have been developed, and the performance of the test carefully analyzed.

Preparation methods were established for cell surface molecules of rat colon carcinoma tissue, functional as target antigens in a radio immunoassay for tumor associated antibodies. This assay will be used to analyze the correlation between the presence of antibodies of various Ig(sub)classes in serum and different "clinical" situations of tumor growth. And for evaluation of different immunization and immunotherapy procedures.

Two sensitive specificity tests for tumor-reactive antibodies have been developed, and the presence of antibodies against rat colon carcinoma associated antigens has been demonstrated. These tests will be useful in the analysis of cross-reactivities between various tumors, embryonic tissues and normal cells.

The biochemical studies of the colon carcinoma associated antigens have resulted in partial biochemical characterization of these antigens and establishment of methods for their further purification. Knowledge about the biochemical nature of the tumor antigens might give information about the functions of these antigens, and the relationships between these antigens and their possible normal counterparts.

By use of the established methodology, it will now be feasible to analyze the involvement, functions, and importance of antibodies of various immunoglobulin types in the immune response against rat colon carcinoma. Furthermore, a basis has been created for the further investigation of the physiochemical and immunobiological properties of the colon carcinoma associated antigens.

All techniques described in this thesis for use in the analysis of the molecular immunology of rat colon carcinoma might directly be applied to the investigations on human tumors. That is, if the tumor antigens of the neoplasm under study can be obtained in soluble form, and functional tumor immune sera are available. Studies by others on human sarcomas have indicated this feasibility (65).

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FRACTIONATION OF RAT IgG SUBCLASSES AND SCREENING FOR IgG Fc-BINDING TO BACTERIA

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Abstract—The four IgG subclasses of the rat, IgG1, IgG2a, IgG2b and IgG2c, were purified from normal serum by a combination of protein A-affinity chromatography and DEAE-cellulose chromatography. Purified, radiolabelled preparations of IgG were tested for binding to Gram-positive bacteria representing five different Fc-receptor (FcR) types. Distinct rat subclass-specific Fc-binding was noted to bacterial species belonging to different Fc-receptor types. *Staphylococcus aureus* (FcR I) strains bind IgG1 and IgG2c as shown by others. Group C and G Streptococci (FcR III) bind all four subclasses of rat IgG. *Streptococcus zooepidemicus* strains (FcR V) also bind all four subclasses but only to a lower degree. Human group A Streptococci (FcR II) and bovine group G Streptococci (FcR IV) do not bind any of the rat IgG subclasses.

Elution studies on two strains, *Staphylococcus aureus*, Cowan I, and human group G Streptococcus, G 148, showed that both thiocyanate and pH-elution might be useful for the fractionation of IgG subclasses bound to bacterial cells. The present work indicates the possible use of bacterial cells as solid-phase absorbents in immunological studies of rat IgG.

INTRODUCTION

It is of great importance to develop techniques and reagents which make it possible to evaluate the role of a given antibody population in complex immune phenomena and investigate in detail the biological characteristics of immunoglobulin classes and subclasses. A major problem is that various purification and fractionation procedures may partially or totally destroy the biological activity of the immunoglobulins.

One widely used reagent for immunoglobulin studies in various species is the immunoglobulin Fc-binding surface component, protein A, of *Staphylococcus aureus* [for review see Goding (1978)]. It has been used for studies of the humoral response against tumors in rats (Steele *et al.*, 1974) and in humans (Steele *et al.*, 1975). Five different classes of immunoglobulins are recognized in the rat with designations according to Bazin *et al.* (1974): IgG, IgM, IgA, IgD and IgE. IgG contains four different subclasses: IgG1, IgG2a, IgG2b and IgG2c. *Staphylococcus aureus* has earlier been shown to bind two rat IgG subclasses, IgG1 and IgG2c (Medgyesi *et al.*, 1978).

Several species of Streptococci have been shown to carry Fc-binding structures analogous to protein A (Kronvall, 1973). Previous studies have shown that this binding is mediated by five major types of Fc-receptors (FcRs) including protein A (Myhre & Kronvall, 1977, 1980a, b; Myrhe *et al.*, 1979).

The purpose of the present work was to investigate the possible existence of distinctive rat subclass specificities within Fc-binding bacterial species with different FcR types. The definition of specificities different from that of protein A should increase the potential of using such Fc-affinity reagents as tools in studies of humoral immune responses in rats.

MATERIALS AND METHODS

Source of rat serum

Normal untreated inbred Wistar/Furth female and male rats of various ages were bled by cardiac puncture under ether anaesthesia and serum collected and stored at -20°C .

Fractionation of rat serum on protein A-Sepharose with sodium thiocyanate elution

Untreated serum (25 ml) was applied to a column (0.9×6.0 cm) with protein A-Sepharose CL 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBSG (10 mM

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sodium phosphate, 0.15 *M* sodium chloride, 0.1 *M* glycine, pH 7.3). Elution was performed at 12 ml/hr and 3-ml fractions were collected. When the absorbance at 280 nm reached baseline, a wash with 1.0 *M* NaCl in PBSG adjusted to pH 6.0 was made to remove contaminating IgG2a and IgG2b, followed by a wash with PBSG before elution was started with 0.5 *M* NaSCN in PBSG to obtain IgG1. Elution continued until the absorbance reached the base line, after which IgG2c was eluted with 1.0 *M* NaSCN. Immediately after elution, fractions corresponding to a peak were pooled and desalted on Sephadex G-25 medium in a column (2.5 × 30 cm) equilibrated with PBSG at a flow rate of 80 ml/hr.

DEAE-cellulose chromatography

Fractionation of rat immunoglobulin was performed on DEAE-cellulose (Whatman DE 52) by stepwise elution with increasing salt concentrations (Medgyesi *et al.*, 1978). The immunoglobulin fraction of serum or fractions from protein A-Sepharose chromatography were precipitated at 4°C by the addition of saturated ammonium sulphate solution to a final saturation of 40%. After centrifugation at 12,000 *g* for 15 min (Sorwall SS-34, 15,000 rev/min) the precipitate was dissolved in the original volume of 0.15 *M* NaCl and dialysed overnight against 5 mM sodium phosphate buffer, pH 8.0, centrifuged and about 400 mg of protein was then applied to a column (1.5 × 30 cm) of DEAE-cellulose equilibrated with this buffer. Stepwise elution was performed with sodium phosphate buffers, pH 8.0, with molarities of 5, 15, 50 and 300 mM at a flow rate of 25 ml/hr. Five-millilitre fractions were collected. Fractions corresponding to each of the peaks were pooled and dialysed against PBSG, pH 7.3, and stored at -20°C. The fraction eluted with 5 mM phosphate buffer, pH 8.0, contained pure IgG2a. A mixture of IgG2a and IgG2b was eluted with 0.015 *M* buffer. The 0.050 *M* eluate contained IgG2a, IgG2b and residual IgG1. IgM together with IgG1, IgG2a and IgG2b were eluted with 0.3 *M* phosphate buffer.

Protein A-Sepharose affinity chromatography with pH-elution at low pH

Proteins eluted from DEAE-cellulose with 15 mM phosphate buffer, pH 8.0, were pooled and concentrated in an Amicon stirred cell over a PM 10 membrane. After dialysis against 0.14 *M* sodium phosphate buffer, pH 8.0, 100 mg of

protein was applied onto a protein A-Sepharose column (0.9 × 6.0 cm) equilibrated with the same buffer. After the unbound fraction, containing IgG2a and IgG2b, had been washed off the bound fraction was eluted stepwise with 0.14 *M* phosphate buffers, pH 7.0 and 6.0. In some separations the pH 6-fraction contained small amounts of IgG1, in addition to IgG2b but the pH 7-fraction contained exclusively IgG2b.

Immunodiffusion techniques

The immunoglobulin content of various fractions was analysed with radial double immunodiffusion performed in 1% agar gel (agar for electrophoresis, BDH, Poole, U.K.) in PBS pH 7.3. Monospecific antisera against rat immunoglobulin classes and subclasses were obtained commercially (Miles Laboratories Inc., U.K.). These antisera were checked for cross-reactivity by double immunodiffusion and for reactivity against non-Ig serum proteins in immunoelectrophoresis against rat serum and were shown to be specific. The detection limits for the different immunoglobulins, as determined by end point dilution of normal serum, were: for IgG1, 1.5%; for IgG2a, 0.4%; for IgG2b, 0.4%; for IgG2c, 3%; for IgM, 3%; and for IgA, 3%, of the concentration in serum. Immunoelectrophoresis was carried out according to Grabar & Williams (1953) with 1% agar in 10 mM barbital, 50 mM sodium barbital, 50 mM sodium acetate, pH 8.6. An anti-rat whole serum pooled from rabbits immunized with rat serum to give a polyvalent antiserum was used for immunoprecipitation.

Radiolabelling of immunoglobulin preparations

Purified immunoglobulin preparations were ¹²⁵I-labelled (code No. IMS 30, The Radiochemical Centre, Amersham, U.K.) by use of lactoperoxidase coupled covalently to Sepharose (David & Reisfeld, 1974). The degree of incorporation of isotope into protein was estimated by paper chromatography (Harboe & Fölling, 1974). Free, unreacted isotope was removed by extensive dialysis against PBSA (phosphate buffered saline: 0.12 *M* NaCl, 0.03 *M* phosphate, pH 7.3, containing 0.05% Tween 20). Labelled preparations showed specific activities of 10–20 MBq per milligram of protein (1 mCi = 37 MBq). Twenty-five microlitres used in tests contained 0.1 µg of radiolabelled protein. The labelled protein was used within 1 month. A lower uptake was observed after storage for longer time periods.

Bacterial strains and cultivation

Sixty-nine strains were included in the screening for binding activity against the Fc-part of rat IgG: 10 strains of *Staphylococcus aureus* 10 group A Streptococci, 10 *Streptococcus equisimilis*, 10 *Streptococcus zooepidemicus*, nine *Streptococcus dysgalactiae*, 10 human group G Streptococci and 10 bovine group G Streptococci. Staphylococci were grown in tryptone broth and Streptococci were cultured in Todd-Hewitt broth. After 16 hr of incubation at 37°C, the bacteria were harvested and washed twice in PBSA. The optical density at 620 nm was measured (Beckman model CP-1), and the bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms/ml.

Quantitative binding assay of rat IgG to bacterial cells

The four subclasses of rat IgG were tested for binding to various bacterial strains, by a method described previously for human immunoglobulins (Myhre & Kronvall, 1977). These tests were carried out in polystyrene tubes (12 × 70 mm, Cerbo, Trollhättan, Sweden) by adding 2×10^8 bacteria (200 μ l of a suspension containing 10^9 test organisms/ml) to 25 μ l (0.1 μ g) of labelled IgG. After 1 hr at room temperature, 2 ml of PBSA, pH 7.2, containing 0.05% Tween 20 were added to each tube and the bacteria were spun down. The radioactivity in the pellet was measured in a gamma counter (LKB Rack Gamma model 1270, Biotec., Stockholm). The binding is expressed as percentage of total radioactivity added. Each fraction was run in duplicate. A less than 10% uptake was considered as negative.

Elution from bacterial cells

Radiolabelled subclasses of rat IgG (25 μ l) were allowed to bind to 2×10^8 bacteria of a *Staphylococcus aureus* strain (Cowan I) and a human group G Streptococcus (G 148) in PBSA + 0.05% Tween 20, pH 7.2. The radioactivity of the bacterial pellets was determined and the pellets were then resuspended in 1.0 ml of various elution buffers. After incubation for 30 min at room temperature the bacteria were again sedimented and the radioactivity of the pellets was measured. The amount of IgG still bound was expressed as percentage of radioactivity before elution.

For SCN-elution NaSCN solutions with

molarities ranging from 0.2 to 1.8 M in PBSA + 0.05% Tween 20, pH 7.2, were used. For pH-elution glycine-buffered saline (GBSA: 0.1 M glycine, 0.1 M NaCl, 0.02% sodium azide, 0.05% Tween 20), pH 2–4 and 9–11, or phosphate buffer (0.14 M sodium phosphate, 0.02% sodium azide, 0.05% Tween 20), pH 5–8, was added to the pellets.

RESULTS

Fractionation of rat IgG subclasses

The four subclasses of rat IgG were isolated from normal serum by sequential fractionations (Fig. 1). When possible, 0.1 M glycine was added to decrease aggregation of highly purified immunoglobulins (Hansson, 1970).

By use of immunodiffusion tests the subclass preparations were demonstrated to contain the respective subclass without any contamination of other immunoglobulins. The preparations showed one precipitation arc in immunoelectrophoresis when tested against anti-whole rat serum. The recovery of the different subclasses could not be calculated, as no quantitation method was available.

Quantitative binding assay

Seven different bacterial species, representing five different IgG FcR types, were tested for

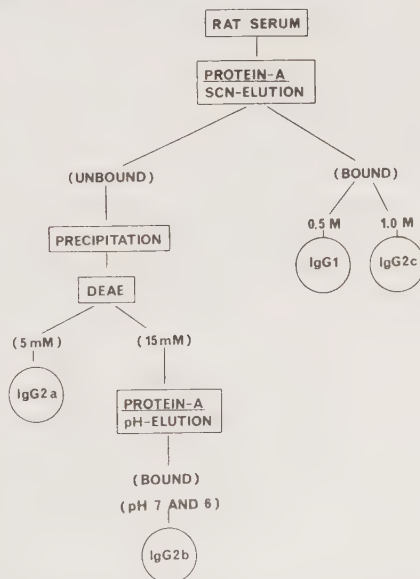


Fig. 1. Flow chart for fractionation of rat IgG subclasses from normal rat serum by protein A-Sepharose chromatography with SCN- and pH-elution and DEAE-cellulose chromatography.

Table 1. Binding of radiolabelled rat immunoglobulin G to different bacterial species

Species	Binding of rat				
	FcR-type*	IgG1	IgG2a	IgG2b	IgG2c
<i>Staphylococcus aureus</i>	I	+	—	—	++
Human group A Streptococci	II	—	—	—	—
<i>Streptococcus equisimilis</i> (human group C)	III	+	++	+	++
<i>Streptococcus dysgalactiae</i> (bovine group C)	III	+	+	+	++
Human group G Streptococci	III	+	++	+	++
Bovine group G Streptococci	IV	—	—	—	—
<i>Streptococcus zooepidemicus</i> (equine group C)	V	+	+	+	+

*IgG Fc-receptor type.

— = uptake less than 10%.

+ = uptake 10–40%.

++ = uptake more than 40%.

binding of radiolabelled rat IgG subclasses. Five different patterns were seen as summarized in Fig. 2 and Table 1.

Staphylococcus aureus [FcR I, Myhre & Kronvall (1977)] selectively binds IgG1 (37% uptake) and IgG2c (70%) and shows no binding of IgG2a (4%) or IgG2b (8%) (Fig. 3A). The 10 different strains of *Staphylococcus aureus* tested demonstrated very little variation in binding capacity and thus seem to form a homogeneous group. One strain (Cowan I) was also tested for binding of rat IgG at pH 8.0, but no difference was detected compared to uptake at pH 7.2 (data not shown).

No strain of human group A Streptococci [FcR II, Myhre & Kronvall (1977)] or bovine group G Streptococci [FcR IV, Myhre *et al.*

(1979)], showed uptake of any of the rat IgG subclasses (Fig. 3F and G). Three different species of Streptococci carrying IgG FcR type III (Myhre & Kronvall, 1977) were tested. *Streptococcus equisimilis* (Fig. 3B) and human group G Streptococci (Fig. 3D) bind all of the four IgG subclasses with a higher uptake of IgG2a (56 and 50%) and IgG2c (67 and 65%) compared to IgG1 (28 and 35%) and IgG2b (35 and 31%). *Streptococcus dysgalactiae* also binds the four subclasses, but with a significantly lower uptake of IgG2a (14%) than the other two Streptococcal species of the FcR III type.

There was a great variation in the binding capacity among the different strains of human group C (*Streptococcus equisimilis*) and human group G Streptococci. However, no strain

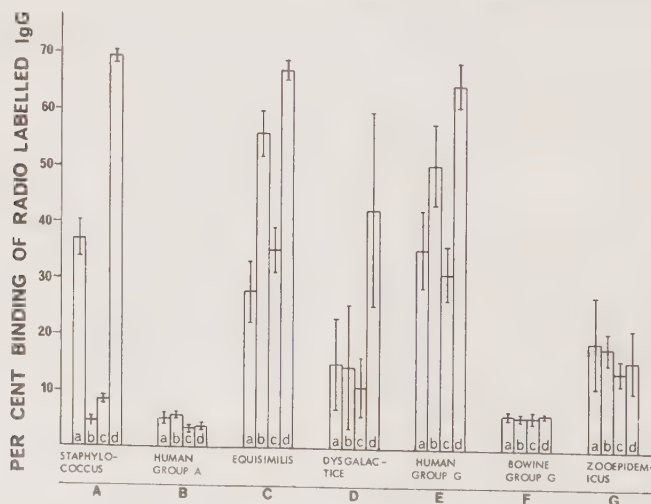


Fig. 2. Binding of purified rat IgG subclasses to 69 strains of Fc-binding bacteria. (a) IgG1; (b) IgG2a; (c) IgG2b; (d) IgG2c. *Staphylococcus aureus* (A); human group A Streptococci (B); *Streptococcus equisimilis* (C); *Streptococcus dysgalactiae* (D); human group G Streptococci (E); bovine group G Streptococci (F); *Streptococcus zooepidemicus* (G). The figure shows the mean and confidence interval ($P = 0.05$) of the uptake of IgG to the bacterial species. The binding capacity is expressed as percentage of added activity.

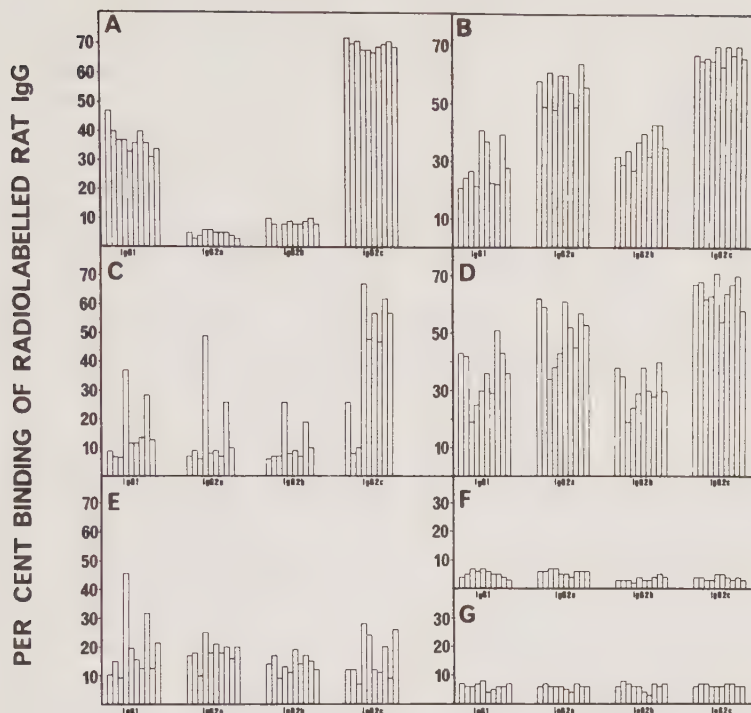


Fig. 3. Binding of rat IgG subclasses to 69 strains of Fc-binding bacteria. Radiolabelled IgGs (0.1 μ g) were allowed to bind to 2×10^8 bacteria. The binding capacity of individual strains is expressed as percentage of added activity. *Staphylococcus aureus* (A); *Streptococcus equisimilis* (B); *Streptococcus dysgalactiae* (C); human group G Streptococci (D); *Streptococcus zooepidemicus* (E); human group A Streptococci (F); bovine group G Streptococci (G).

showed preferential binding of any particular IgG subclass. Strains with high binding levels of one subclass also bound the other subclasses to a higher degree. *Streptococcus zooepidemicus*, which has recently been found to carry a new type of FcR [FcR V, Myhre & Kronvall (1980a)], binds all four subclasses of rat IgG to a low degree (14–20% uptake) (Fig. 3D).

Elution studies

One *Staphylococcus aureus* strain (Cowan I) and one strain of human group G Streptococci (G 148) were used for elution studies with thiocyanate- and pH-elution. pH-elution was studied at both low and high pHs.

With *Staphylococcus aureus* higher concentrations of thiocyanate were needed to elute IgG2c than IgG1 (Fig. 4A). Analogously, a greater change of pH (Fig. 5C and D) was required for elution of IgG2c compared to IgG1. A pH below 5 can not be used for *Staphylococcus aureus*, since it causes aggregation of the bacteria. IgG2a and IgG2b do not bind to *Staphylococcus aureus*.

The G 148 strain binds all four rat IgG subclasses, which can subsequently be eluted with thiocyanate. IgG1 and IgG2a showed a very low affinity binding and were eluted with 0.2 M SCN (Fig. 4B). IgG2b binds with slightly higher affinity, whereas elution of IgG2c from G148 required higher concentrations compared to those necessary for *Staphylococcus aureus*. An analogous distinct difference between IgG2c and the other subclasses was observed using pH-elution (Fig. 5A and B).

DISCUSSION

Serum IgG of untreated rats was fractionated into the four subclasses IgG1, IgG2a, IgG2b and IgG2c by a combination of affinity chromatography on protein A-Sepharose and ion exchange chromatography on DEAE-cellulose.

IgG1 and IgG2c were separated by a rather mild technique without any pretreatment of the serum; the possible denaturation effect of NaSCN is greatly inhibited by stabilization of

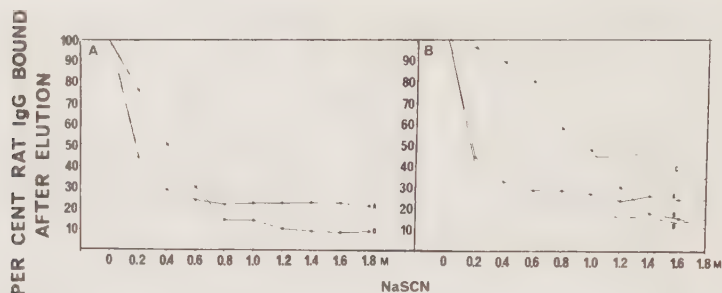


Fig. 4. Elution of rat IgG bound to *Staphylococcus aureus* Cowan I (A) and human group G *Streptococcus* G 148 (B) with increasing concentrations of SCN. (A) IgG1; (B) IgG2a; (C) IgG2b; (D) IgG2c. The amount of radioactivity remaining on the bacteria after the elution is plotted as percentage of the radioactivity initially bound to the bacteria.

the IgG fractions with 0.1 M glycine added to the elution buffers. It should be noticed that low concentrations of thiocyanate were used compared to the standard use of 3 M SCN in general immunosorbent separations. The ion exchange step used for fractionation of IgG2a and IgG2b includes precipitation with ammonium sulphate as a possible denaturing step. The IgG2a and the IgG2a/IgG2b fraction were subsequently eluted from DEAE-cellulose without the use of extreme pH or high ion concentrations. The protein A separation of IgG2b did not involve the use of extreme pH.

The present studies of IgG binding to components on the bacterial cell surface of Gram-positive cocci were performed using a well-established assay technique (Kronvall *et al.*, 1970). The sensitivity of the test procedure

depends on the ratio between the quantity of IgG and the number of bacterial organisms. All tests were carried out with an excess of bacteria. Under these circumstances almost all of the reactive IgG molecules will bind to the bacterial surface when the binding affinity is high. The immunochemical aspects of binding have been studied with human IgG subclasses (Myhre & Kronvall, 1980b).

The demonstrated binding of rat IgG subclasses to *Staphylococcus aureus* strains is in accordance with previous studies on the binding of rat myeloma proteins and IgG fractions of serum to the *Staphylococcus aureus* strain Cowan I (Medgyesi *et al.*, 1978). In these studies it was also demonstrated that protein A binds to both IgG1 and IgG2c but precipitates only IgG2c. In the present investigation binding of rat IgG



Fig. 5. Elution of rat IgG bound to *Staphylococcus aureus* Cowan I (C and D) and human group G *Streptococcus* G 148 (A and B) by change of pH. (A) IgG1; (B) IgG2a; (C) IgG2b; (D) IgG2c. Both an increase (A and C) and decrease (B and D) of pH were used. The amount of radioactivity remaining on the bacteria after the elution is plotted as percentage of the radioactivity initially bound to the bacteria.

subclasses to *Staphylococcus aureus* Cowan I was studied at pH 8.0 and 7.2 as well. In contrast to the increased binding of IgG2b to protein A-Sepharose at high pH no increase in the binding to Cowan I was seen at pH 8.0 for any of the subclasses. This may depend on differences in steric or other molecular properties between the protein A at bacterial surfaces and the protein A bound to Sepharose. A non-specific binding of IgG2b to agarose may also be responsible for this effect. However, this have not been seen during the use of other separations involving a Sepharose-based affinity matrix.

Two of the three tested Streptococcal species with FcR type III (*Streptococcus equisimilis* and human group G Streptococci) behaved very similarly with binding of all the four subclasses with a higher uptake of IgG2a and IgG2c compared to IgG1 and IgG2b. The third species *Streptococcus dysgalactiae*, had a similar pattern, but with a significantly lower uptake of IgG2a. This may indicate that *Streptococcus dysgalactiae* has a new FcR type different from FcR type III. FcR type II (human group A Streptococci) and FcR type IV (bovine group G Streptococci) were shown not to bind rat IgG. FcR type V (*Streptococcus zooepidemicus*) has a low uptake of all subclasses.

Two strains, *Staphylococcus aureus* Cowan I and the human group G Streptococcus G 148, were selected for investigations of the usefulness of IgG Fc-binding bacteria for preparative purposes. When elution experiments were performed on *Staphylococcus aureus* Cowan I with thiocyanate or pH-elution, the results were as expected from experiments on protein A-Sepharose. It is shown that IgG2c binds with a higher affinity than IgG1. This is true also in protein A-Sepharose chromatography, where it is possible to elute bound IgG1 with IgG2c. However, it should be noticed that IgG2c is eluted with a relatively low concentration of thiocyanate compared to that required to elute human IgG bound to protein A.

All four subclasses bind to the human group G Streptococcal strain G 148, but the elution studies show that except for IgG2c most of the IgG is rather weakly bound. This shows that the strain G 148 can be used mainly for IgG2c absorption and purification.

The studies of elution by alteration of pH showed that increase of pH has a higher potential for separation of bound IgG than similar gradient elution at low pH.

This work has demonstrated the existence of

distinctive rat IgG subclass specificities different from the protein A pattern, within various species and strains of IgG Fc-binding Streptococci. It has also indicated that it may be possible to fractionate the Ig bound to the bacterial surfaces by use of standard elution techniques. Work is in progress in our laboratories to define techniques which make it possible to use bacterial cells as solid-phase absorbents for various analytical and preparative purposes.

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Different Protein A Immunosorbents May Have Different Binding Specificity for Rat Immunoglobulins

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Purified polyclonal immunoglobulin preparations representing the 4 rat IgG subclasses were tested for binding to *Staphylococcus aureus* protein A attached to 3 different solid phases (*Staphylococcus aureus* Cowan I bacteria, Sepharose CL4B and Sepharose 6MB). Protein A Sepharose CL4B showed higher reactivity with IgG1 and IgG2b than the staphylococci, whereas protein A Sepharose 6MB showed a lower uptake of these subclasses. No differences were seen for IgG2a and IgG2c. Protein A on different solid phases cannot be used interchangeably without confirmation of binding specificity.

Key words: protein A-Sepharose — protein A macrobeads — rat IgG subclasses — immunosorbents

Introduction

Protein A, the immunoglobulin binding surface component of *Staphylococcus aureus*, is a widely used reagent for immunoglobulin studies. Intact *S. aureus* organisms can be used for solid-phase absorption of immunoglobulins (Goding, 1978).

Protein A covalently linked to defined matrices is commercially available and has to a large extent replaced the use of whole staphylococcal organisms. However, binding of soluble proteins to ligands linked to solid phases is dependent on the microenvironment as well as the nature of the attachment to the solid matrices (Turkova, 1978). The binding characteristics of protein A linked to various matrices may vary and may differ from the interaction with protein A exposed on the bacterial cell surface.

The object of this study was to investigate whether *S. aureus* organisms and protein A-Sepharose gels may be used interchangeably as immunosorbents. Purified

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rat immunoglobulins representing the 4 IgG subclasses were used because these subclasses differ markedly in protein A reactivity making it possible to detect even small differences in specificity.

Materials and Methods

Fractionation of rat IgG subclasses

Normal inbred Wistar/Furth female and male rats of various ages were bled by cardiac puncture under ether anaesthesia and serum collected, pooled and stored at -20°C . Serum was fractionated into the 4 subclasses IgG1, IgG2a, IgG2b, and IgG2c by a combination of affinity chromatography on protein A Sepharose CL4B and ion exchange chromatography on DEAE cellulose as previously described (Nilsson et al., 1982b). The identity and purity of each IgG subclass preparation was confirmed in double immunodiffusion tests performed with monospecific rabbit antisera against rat subclasses (Nilsson et al., 1982a). The preparations showed single precipitation arcs in immunoelectrophoresis when tested against an anti-whole rat serum reagent.

Radiolabelling of immunoglobulin preparations

Purified immunoglobulins were ^{125}I -labelled (code no. IMS 30; The Radiochemical Centre, Amersham) by the lactoperoxidase method (David and Reisfeld, 1974). Labelled preparations showed specific activities of 10–20 MBq per milligram of protein. The labelled protein was used within 1 month. A lower uptake was observed after storage for longer time periods.

Solid phases

Protein A Sepharose CL4B, protein A Sepharose 6MB (macrobeads), Sepharose CL4B and CNBr-activated Sepharose 6MB were obtained from Pharmacia Fine Chemicals (Uppsala). Protein A from an identical source was coupled to the 2 types of Sepharose by use of exactly the same cyanogen bromide technique (Porath et al., 1967; Per Vretblad, Pharmacia, personal communication).

Ten different strains of *Staphylococcus aureus* were randomly collected from the clinical specimens received at the Clinical Microbiology Laboratory, University Hospital, Lund. Staphylococci were cultured at 37°C for 16 h in tryptone soya broth. The bacteria were then deposited by centrifugation and washed twice in PBSA (10 mM sodium phosphate, 0.15 M sodium chloride, 0.02% sodium azide, pH 7.2). The optical density at 620 nm was measured (Beckman model CP-1). The bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms/ml. Freshly grown bacteria were used in the tests.

Quantitative binding assay

Immunoglobulin preparations representing the 4 rat IgG subclasses were tested for binding to the different solid phases, by a modification of the method described

previously for human immunoglobulins binding to bacterial cells (Myhre and Kronvall, 1977).

These tests were carried out in duplicates in polystyrene tubes by adding 2×10^8 bacteria or 100 μ l sedimented Sepharose gel to 0.1 μ g of 125 I-labelled IgG in a total volume of 225 μ l PBSA containing 0.05% Tween 20 (PBSA-Tween). The immunoglobulin binding capacity of the amount of protein A used in the experiments is approximately 5 mg for the bacteria, 2.5 mg for protein A Sepharose CL4B and 1.25 mg for protein A Sepharose 6MB. The tubes were rocked for 2 h at room temperature. The suspensions were then washed 4 times with 2 ml PBSA-Tween, and the bound radioactivity measured in a gamma counter (Beckman Gamma 310 system, Stockholm). The binding was expressed as percentage of total radioactivity added. An uptake less than 10% was considered negative.

Results

Purified, polyclonal immunoglobulin preparations representing the 4 rat IgG subclasses were tested for binding to protein A attached to 3 different matrices. Distinct differences in binding specificity were observed (Fig. 1). The discrepancies were most pronounced for IgG1 and IgG2b. Protein A Sepharose CL4B showed

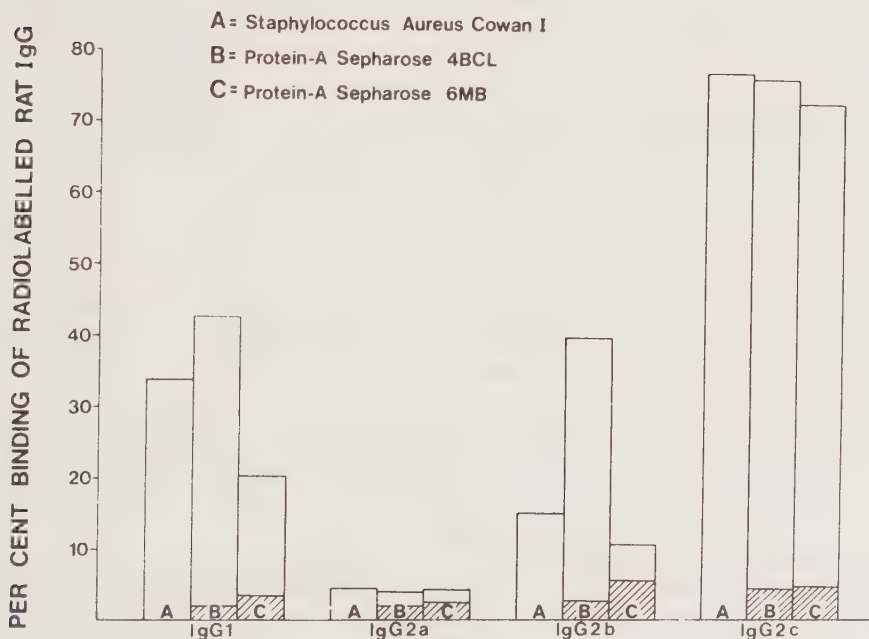


Fig. 1. Uptake (%) of radiolabelled rat immunoglobulin G subclasses by protein A attached to different matrices. Shaded bars indicate binding to Sepharose without protein A.

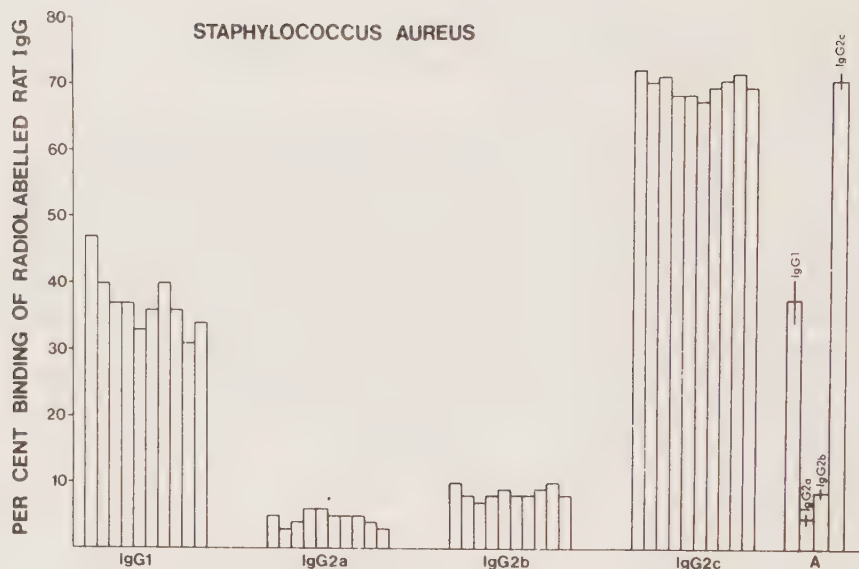


Fig. 2. Binding of purified rat IgG subclasses to 10 strains of *Staphylococcus aureus*. A, mean and confidence interval ($P = 0.05$) for the 10 strains.

higher uptake of these subclasses than *S. aureus*, and protein A Sepharose 6MB showed a lower uptake. No difference in reactivity for IgG2a and IgG2c was detected.

Ten different *Staphylococcus aureus* strains were studied. They demonstrated very little variation in binding pattern and thus form a homogeneous group (Fig. 2). Low but detectable uptake of IgG2b was observed.

Discussion

Purified radiolabelled rat immunoglobulin G subclasses were shown to form a sensitive system for analysis of the binding specificity of protein A. The different subclasses represent immunoglobulins with high (IgG2c), intermediate (IgG1), low (IgG2b) and no (IgG2a) reactivity with protein A.

Significant differences in the binding of IgG1 and IgG2b were detected when the 2 types of protein A Sepharose gels were compared with whole *S. aureus* bacteria. Protein A Sepharose CL4B showed a higher and protein A Sepharose 6MB a lower uptake of these subclasses than the staphylococci.

The present studies were performed with a well-established assay technique (Kronvall et al., 1970; Myhre and Kronvall, 1980). All tests were carried out with 0.1 μ g labelled IgG and a quantity of protein A capable of binding 1.25 to 5 mg IgG, i.e., with a great excess of immunoglobulin binding sites. The binding of IgG to

staphylococci carrying protein A is a time-dependent process and is complete within 5–10 min (Myhre and Kronvall, 1980). The incubation time of 2 h used in the present binding experiments was sufficient for the binding to reach an equilibrium. Under the experimental conditions used the differences in binding levels observed with protein A attached to different solid phases are therefore of a qualitative nature, mainly reflecting differences in avidity.

Protein A interacts with immunoglobulins via 2 non-immune mechanisms, the classical F_c -mediated binding (Forsgren et al., 1966; Kronvall et al., 1970) and the newly described alternative, $F_{(ab')_2}$ -mediated binding (Inganäs et al., 1980). The mode of covalent linkage of protein A to a solid matrix may influence the availability and nature of the binding sites expressing these 2 reactivities. The differences in porosity between the matrices, which mainly influence reactions with low binding affinity, may also contribute to the effects.

Ten different *S. aureus* strains studied displayed consistent behaviour. In contrast to previous studies (Medgyesi et al., 1978; Nilsson et al., 1982b) weak protein A reactivity was detected with polyclonal IgG2b. This finding was further supported by the positive binding to protein A Sepharose CL4B. The IgG2a subclass was consistently negative with all 3 protein A preparations, indicating complete non-reactivity.

The reasons for the differences in reactivity found between different protein A matrices were not further evaluated. However, the results indicate that the various solid protein A matrices cannot be used interchangeably without confirmation of binding specificity.

Acknowledgements

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Quantitative Analysis of Rat Ig (Sub)classes Binding to Cell Surface Antigens

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An indirect ¹²⁵I-labeled protein A assay for detection of cell surface-bound rat immunoglobulins is presented. The assay is quantitative and rapid and detects as little as 1 ng of cell surface-bound Ig. It discriminates between antibodies belonging to different IgG subclasses, IgM and IgA. We describe the production and specificity control of the reagents used and show that the test can be used for quantitative analysis. A large number of sera from untreated rats are tested to evaluate the frequency of falsely positive responses and variation due to age, sex and strain of rat. With this test it is relatively easy to quantitate the binding of classes and subclasses of rat immunoglobulins in a small volume (6 µl) of untreated serum.

Key words: *rat Ig (sub)classes — protein A — cell surface antigens — binding test — monoclonal Ig*

Introduction

A variety of techniques exists for measuring antibody reactivity against cell surface antigens on intact living cells. Several of these techniques measure a special function of the antibodies, e.g., resulting in lysis of the cells (complement dependent lysis or antibody dependent cellular cytotoxicity) or formation of aggregates or rosettes (hemadsorption and immune adherence). These functional tests are limited to qualitative analysis or quantitation by end point dilution. They detect only a subpopulation of the antibodies and provide only limited opportunities to study different classes or subclasses of immunoglobulins directed against the cell surface antigens.

Various antibody-binding assays have been used, which do not measure any effects of antibodies but only the binding. These include immunofluorescence assays (direct or indirect tests), isotopic anti-immunoglobulin tests and labeled (enzyme or isotope) *Staphylococcus aureus* protein A (SpA) tests (with or without a second antibody step). The usefulness of these binding assays is dependent on the reagents

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used. With direct SpA tests only protein A-binding antibodies are detected and when secondary antibodies are used, the results are mainly dependent on the characteristics of these antibody reagents.

We describe here an assay which uses ^{125}I -labeled protein A and rabbit antisera as second antibodies to measure rat immunoglobulins of various classes and IgG subclasses binding to surface antigens of cells from solid tumors. Target cells are tested as adherent monolayers in microtest plates.

Materials and Methods

Animals

Rats of the strains Wistar/Furth (WF) and Brown Norway (BN) were used. They were maintained by continuous, single-line, brother-to-sister mating and were kept on a standard pellet diet and water ad libitum.

Tumors and cells

The colon adenocarcinoma DMH-W49 was induced in a WF female rat by 1,2-dimethylhydrazine (DMH). It was kept in serial passage in syngeneic rats. A cell line was established in tissue culture from cells obtained from pieces of tumor tissue exposed to the proteolytic enzyme Dispase II (Boehringer and Mannheim, Stockholm), 2.4 mg/ml for 30 min at room temperature with continuous stirring. The cells were subcultured by trypsinization more than 20 times before used in assays. Cells were grown in Waymouth's culture medium supplemented with 20% fetal calf serum (FCS) and 4 mM L-glutamine. The rat myeloma cell line Y3 Ag 1.2.3 and the mouse myeloma line P3-NS1/1 Ag4-1 (NS-1) were obtained from Dr. Milstein, Cambridge. Myeloma cells were grown in RPMI 1640 supplemented with 10 mM Hepes buffer and 15% (NS-1) or 5% (Y3) FCS, 4 mM L-glutamine and 1 mM sodium pyruvate. All tissue-cultured cells were regularly checked for contamination with mycoplasma and other contaminants by fixation and staining for DNA with the fluorescent dye Hoechst 33258 as previously described (Chen, 1977).

Sources of rat serum

Pooled sera of normal, untreated inbred WF female and male rats of various ages were used for isolation of immunoglobulins. Parts of this serum pool were stored in aliquots and used as normal reference serum (NRS) in the assays.

Sera were also collected from individual, untreated WF and BN rats of various ages and sexes with and without prior starvation for 24 h. These sera were used to determine the binding levels of normal untreated rats. Sera were obtained from tumor-bearing rats and from rats immunized against tumor in various ways. Immunization techniques will be reported in detail elsewhere. Alloantisera (anti-WF and anti-BN) were raised in rats by 3 biweekly intraperitoneal injections of 2×10^7 allogeneic spleen cells. Serum was collected 1 week after the last injection.

Rats were bled under ether anesthesia by cardiac puncture (for serum pools) or by

capillary drainage from the retro-orbital sinus (for individual sera). Sera were separated and stored at -20°C .

Preparation of immunoglobulins from rat serum

The 4 rat IgG subclasses, IgG1, IgG2a, IgG2b and IgG2c, were purified from normal serum by a combination of protein A affinity chromatography with SCN and pH elution and DEAE-cellulose chromatography as previously described (Nilsson et al., 1982).

Production of rabbit antisera against rat immunoglobulins

Antisera containing predominantly IgG antibodies against various rat immunoglobulins were raised in adult outbred rabbits by intramuscular injection of immunoglobulins (0.5–1.0 mg in PBS — 10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.3) emulsified in complete Freund's adjuvant (Difco Laboratories, Michigan, WI). After 1 month the rabbits were boosted intramuscularly with the same amount of immunoglobulin without Freund's adjuvant. Approximately 45 ml of blood was collected from the marginal ear vein on day 5 and 12 after the boost injection. Sera were separated and stored at -20°C . The rabbits were then boosted and serum taken every 8 weeks for up to 1 year. Two rabbits were used for each immunoglobulin type.

The IgG subclasses used for immunization were prepared from rat serum. For immunization against rat IgM and IgA monoclonal immunoglobulins obtained from Y3 Ag 1.2.3 hybridomas were used.

The resultant antisera were absorbed on immunosorbents of immunoglobulins covalently coupled to Sepharose CL 4B (Pharmacia Fine Chemicals, Uppsala) by the cyanogen bromide technique. IgM and IgA columns were prepared using monoclonal immunoglobulins coupled to the solid phase and for IgG subclass columns appropriate IgG fractions of serum were used in the first absorptions and were in later absorptions replaced by monoclonal IgG subclasses. After absorption the antisera were checked for specificity, first with double immunodiffusion and later with a radioimmunoassay.

Immunization, cell fusion and growth of hybridomas producing monoclonal Ig

WF and BN rats were immunized with ovalbumin (Sigma A-5503), allogeneic spleen cells or syngeneic tumor cells (DMH-W49). Spleens were harvested from immunized animals 4 days after the last intraperitoneal booster inoculation. Spleen cells were suspended and fused with rat myeloma or mouse myeloma cells using polyethylene glycol, MW 1500 (Merck-Schuchardt, F.R.G.). The fusion protocol was essentially as described by Galfré et al. (1977, 1979).

After fusion cells were distributed in 24- or 96-well culturing plates (Titertek tissue culture plate, 76-003-05, Flow Laboratories, Stockholm) with 2×10^4 and 3×10^3 rat peritoneal cells as feeder cells, respectively. HAT (13.6 $\mu\text{g}/\text{ml}$ hypoxanthine, 0.176 $\mu\text{g}/\text{ml}$ aminopterin, 3.88 $\mu\text{g}/\text{ml}$ thymidine) was included in the medium from the day of fusion. Ten to 20 days after fusion supernatants were tested by double immunodiffusion technique against 6 different rabbit antisera (directed

against IgG1, IgG2a, IgG2b, IgG2c, IgM and IgA, respectively) and by the indirect ^{125}I -labeled protein A assay for binding to ovalbumin, allogeneic thymocytes or tumor cells, respectively. Selected cultures were cloned twice by the limiting dilution technique (Goding, 1980). One hybridoma cell/well was seeded into 96-well plates with 6×10^5 rat thymocytes/well as feeder cells. By this procedure growth was not observed in more than 30% of the wells in any of the 2 successive clonings. To ensure monoclonality and to exclude variants, Ig class- and subclass-specific antisera were used for characterization of the Ig produced from clones and expanded cultures.

Preparation of monoclonal immunoglobulins

Clones were expanded in large tissue culture flasks or roller bottles. The Ig produced was purified from culture supernatants by different methods depending on the type of Ig and other conditions. Monoclonal IgG1 was purified by passage of supernatant through a protein A-Sepharose column and subsequent elution with 0.1 M glycine/HCl, pH 3.0. IgG2a produced by clones of 2 different hybridomas was found to specifically bind to ovalbumin. Consequently affinity chromatography on ovalbumin-Sepharose was used to purify this immunoglobulin subclass. Supernatant from a culture of mouse (NS-1)-rat hybrid cells producing IgG2b was passaged through a column of rabbit anti-rat IgG2b coupled to Sepharose CL 4B and subsequently eluted with low pH glycine buffer. Rat (Y3)-rat hybrids producing IgG2b, IgG2c, IgM and IgA were readily adapted to growth in serum-free medium supplemented with 5 $\mu\text{g}/\text{ml}$ transferrin and 5 $\mu\text{g}/\text{ml}$ insulin as previously described (Chang et al., 1980). The Ig produced was concentrated by ultrafiltration of one liter of supernatant through an XM-50 membrane (Amicon).

Specificity tests of class- and subclass-specific rabbit anti-rat Ig antisera

An indirect binding assay with ^{125}I -labeled SpA was used with target preparations of monoclonal rat Ig absorbed to the bottom of the wells in 96-well microtiter plates.

Ig preparations were diluted in PBS, pH 7.3, to a final protein concentration of 5 $\mu\text{g}/\text{ml}$ and incubated in volumes of 50 μl for 3 h at 37°C. The plates were washed twice with PBS. Waymouth medium with 20% FCS (W20), 200 $\mu\text{l}/\text{well}$, was added and plates were incubated overnight at 37°C to block remaining plastic binding sites. The medium was aspirated and 50 μl of (sub)class-specific rabbit antiserum diluted in W20 were added. After incubation for 45 min at 37°C and 2 washes with phosphate-buffered saline with 10 mg/ml BSA(WB), 10^5 cpm of ^{125}I -labeled protein A diluted in WB buffer were added. After 45 min incubation at 37°C plates were washed twice with WB buffer and bound radioactivity eluted with 0.1 M glycine/HCl, pH 2.2. Elution buffer was transferred to counting vials and activity counted in an LKB Beckmann gamma counter 310 system.

Preparation of radiolabeled protein A

Staphylococcus aureus protein A (SpA) (Pharmacia Fine Chemicals) was ^{125}I -labeled by use of the lactoperoxidase method (Thorell and Larson, 1978). To 50 μg SpA in 35 μl 0.05 M sodium phosphate, pH 7.3, were added 4.0 μl (1.0 mg/ml) lactoperoxidase (Sigma L-2005, 61 U/mg, E.C. 1.11.1.7), 1.0 mCi Na^{125}I (100

mCi/ml, code IMS 30, The Radiochemical Centre, Amersham) and 10 μ l 0.003% H_2O_2 . After incubation for 15 sec under stirring 0.5 ml phosphate buffer with 10 mg/ml BSA (Sigma A-4503) were added. The reaction mixture was immediately applied on a G25 column (PD-10, Pharmacia Fine Chemicals) presaturated with 10 mg/ml BSA and 0.5 ml fractions were collected. The 2 fractions in the void volume with the highest activity were pooled and used in the assays. The labeled SpA was titrated to find a suitable dilution and the background binding was checked before it was used in assays. The specific activity obtained was $10\text{--}15 \times 10^6$ cpm/ μ g SpA.

Radioimmunoassay for rat antibodies to cell antigens

The assay is a modification of previously reported procedures (Brown et al., 1977; Zeltzer and Seeger, 1977). Target cells were removed from culture flasks with trypsin-EDTA (0.5 mM EDTA (Merck 8418), 1 mg/ml trypsin (SBL Stockholm), in PBS, pH 7.3) and plated into flat-bottomed microtest plates (Falcon Products no. 3042, Oxhard) with 12,000 cells in 0.2 ml W20 medium per well. After allowing 18–24 h for the target cells to adhere, plates were washed once with 0.2 ml W20 medium to remove nonadherent cells and cell products from the confluent monolayers. To initiate the test, the medium was aspirated from the wells and 50 μ l of rat serum diluted 1/100 in W20 medium were added. Each serum was run in 12-fold parallel (for duplicates with 6 different rabbit antisera). After 45 min incubation at 37°C in 5% CO_2 , the sera were aspirated and each well washed twice with 0.2 ml of WB buffer. Then 50 μ l of respective rabbit antiserum diluted in W20 medium were added in duplicates and the plates were again incubated for 45 min at 37°C in 5% CO_2 and washed twice with 0.2 ml of WB buffer. A volume of 50 μ l (about 50,000 cpm, 6 ng) of ^{125}I -SpA diluted in WB buffer was added and incubated for 45 min at 37°C and 5% CO_2 . After 2 washes the bound radioactivity was eluted by adding 100 μ l SDS solution (0.08 M sodium dodecyl sulfate in WB buffer without BSA). After 5 min in room temperature the supernatants were collected with the Titertek supernatant collection system (no. 78-215-99 and 78-210-05, Flow Laboratories, Stockholm). The tubes were counted in the gamma counter for 2 min per tube. Each assay (normally 3 plates) includes one NRS sample, as reference for background and 1 alloantiserum as positive control. The binding is expressed as the ratio between the radioactivity of the test serum and the radioactivity of the normal reference serum pool:

$$\text{antibody binding ratio} = \frac{\text{cpm for test serum}}{\text{cpm for NRS}}$$

Ig-binding levels in serum of untreated rats

Sera from untreated rats of various strains, ages and sexes (Table I) were collected to determine the antibody-binding levels in normal rats and to investigate the frequency of falsely positive sera. Fifty percent of the sera were from starved rats. These sera were analyzed in the binding assays as test sera in a random order and the ratios against NRS were determined. The SPSS (Nie et al., 1977) and BMDP (Dixon, 1977) computer programs were used for data handling and statistical analysis of the results.

Results

Immunization of rabbits against rat Ig

Serum IgG of untreated rats was fractionated into the 4 subclasses, IgG1, IgG2a, IgG2b and IgG2c, by sequential fractionations on SpA-Sephadex and DEAE-cellulose. These immunoglobulin preparations and IgM and IgA produced by hybridoma cultures, were used for immunization of rabbits. As the intact immunoglobulins and not Fc fragments were used, the rabbits were immunized also against the light chains of the Ig. Furthermore heavy chain cross-reactivities were detected.

The antisera were adsorbed on immunosorbents of serum-derived or monoclonal immunoglobulins covalently linked to Sephadex until they showed monospecificity in immunodiffusion tests. When tested against rat serum in double immunodiffusion together with commercially obtained antisera against rat Ig classes and subclasses (Miles Laboratories, England), they showed total identity. When tested in the indirect protein A-binding assay against solid phase monoclonal Ig, as described in Materials and Methods, the antisera were shown still to cross-react and the absorption on immunosorbents had to be repeated. After a varying number of absorptions all antisera were finally shown to be monospecific in the sensitive indirect SpA-binding assay also. Fig. 1 illustrates the results of the specificity tests. Dilutions of rabbit antisera used in the binding assays are presented but specificities were evaluated from the information given by the complete dilution curve.

Quantitative aspects of the test

The quantitative aspects of the test were investigated in 2 different experiments. First the linearity of the response was checked. Sera were diluted in a normal serum pool to give a total serum concentration of 1/25. When tested in the binding assay a linear response was obtained over a broad concentration range (1/25–1/1600) (Fig. 2).

A saturation experiment was also performed in which increasing amounts of monoclonal IgG1 alloantibody was incubated with a constant number of cells and bound Ig quantitated. Fig. 3 shows that the response is only dependent of the first step in the test, that is, the amount of rat Ig. This experiment also shows that the detection limit is 1.5 ng (30 ng/ml). No competition was demonstrable between different immunoglobulin types of highly reactive sera. That is, the relative proportions of individual (sub)classes are the same at various dilutions tested.

Reproducibility and variation

Twenty-four sera of different quantitative and qualitative binding patterns were repeatedly (7 times) tested over a period of 5–6 weeks to analyze the reproducibility of the test. This also included different batches of radiolabeled protein A and the use of 2 different batches of target cells obtained from thawed ampoules. This day-to-day variation was shown to be 10% of the mean of the binding ratio.

The interplate variation was studied by running serum in more than 1 plate in the same test. The intraplate variation was tested by running the same serum in a whole

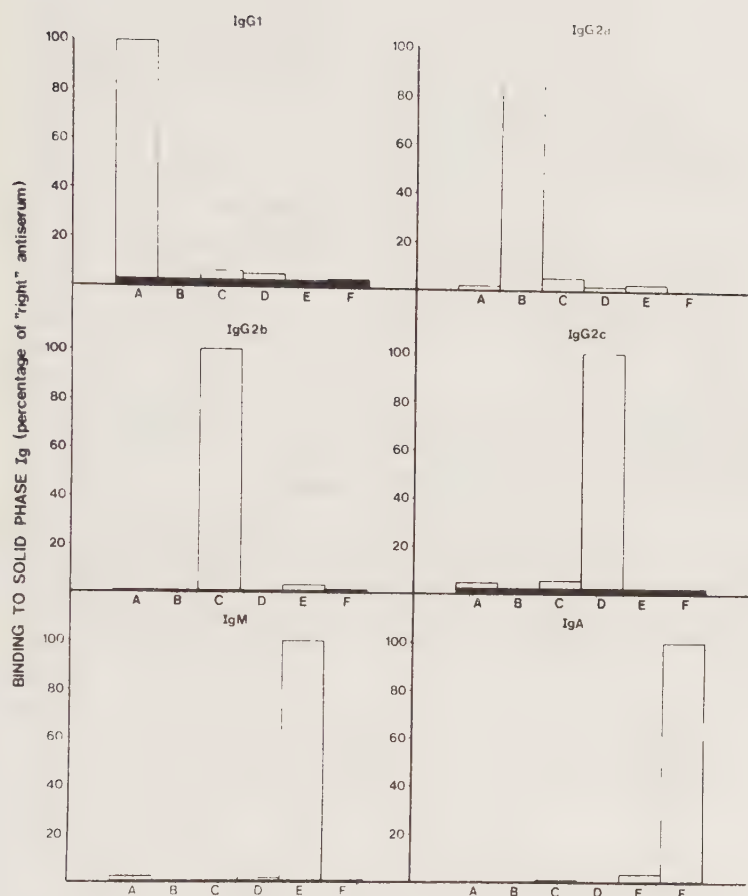


Fig. 1. The specificities of rabbit antisera against classes and subclasses of rat immunoglobulin. The antisera were tested in the indirect ^{125}I -protein A-binding assay using monoclonal immunoglobulins as solid-phase antigen. The bars illustrate the mean bindings to 2 different monoclonal immunoglobulins of each type. Binding is expressed as percentage of 'right' antiserum. Shaded bars illustrate direct binding of SpA to the solid phase. The difference in binding between the 2 monoclonal Igs of the same type was less than 5%. Dilutions are the same as used in subsequent assays. (A = anti-IgG1, B = anti-IgG2a, C = anti-IgG2b, D = anti-IgG2c, E = anti-IgM, F = anti-IgA.)

plate. These inter- and intra-plate variations were both shown to be less than 1% of the mean.

Specificity of the assay

The specificity is illustrated in Fig. 4, showing a criss-cross study of 2 alloantisera tested on both syngeneic and allogeneic target cells. No positive reaction was detected against the syngeneic cells, even if the reaction was very strong against allogeneic target cells.

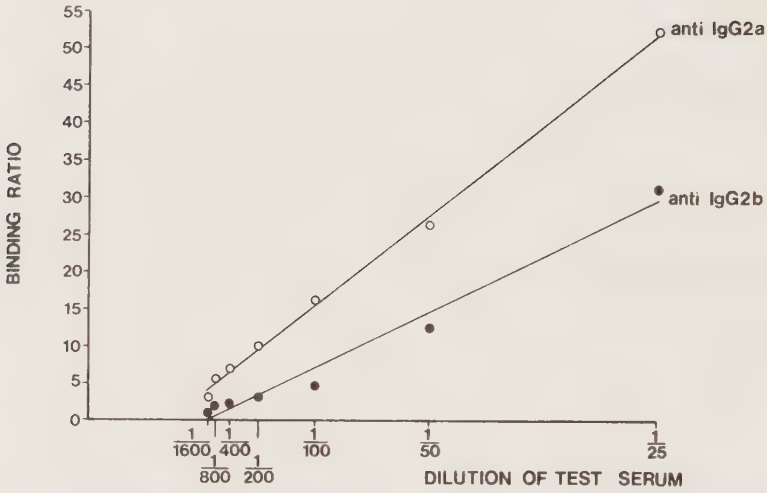


Fig. 2. Binding capacity of various dilutions of a tumor immune serum to DMH-W49 rat colon carcinoma cells. The serum was diluted in normal rat serum as to give a total serum protein concentration of 1/25. The binding ratio was shown by regression analysis to be linear (anti-IgG2a, $r^2 = 0.997$; anti-IgG2b, $r^2 = 0.978$). The serum was obtained from a rat immunized i.p. with tissue cultured W49 tumor cells. Binding is expressed as the ratio between the bound radioactivity for test serum and NRS.

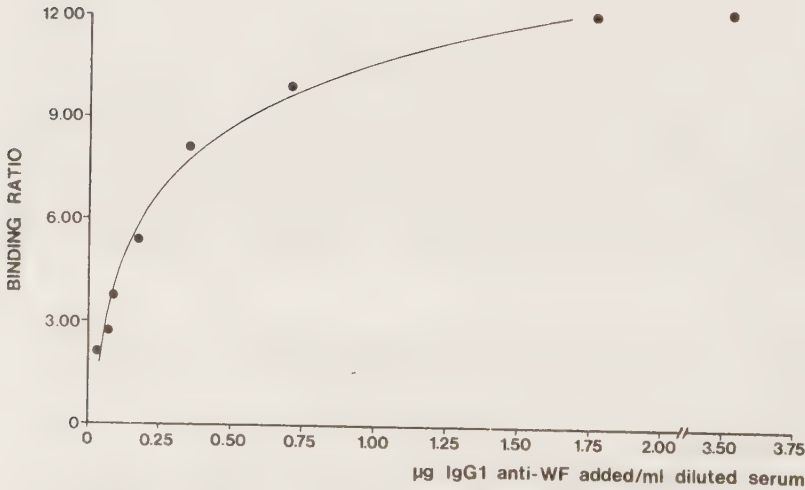


Fig. 3. Saturation curve for a monoclonal IgG1 alloantibody. W49 tumor cells were used as targets. The immunoglobulin was diluted in normal serum diluted 1/100. The binding is expressed as the ratio between the bound radioactivity for serum with and without the IgG1 monoclonal antibody.

TABLE I
Ig-BINDING LEVELS IN NORMAL UNTREATED RATS

Animal group	No. of rats	Binding ratio (mean $\pm$ S.D.)					
		IgG1	IgG2a	IgG2b	IgG2c	IgM	IgA
All rats	126	1.3 $\pm$ 0.4	1.4 $\pm$ 0.5	1.5 $\pm$ 0.5	1.4 $\pm$ 0.4	1.4 $\pm$ 0.5	1.1 $\pm$ 0.5
WF-rats	67	1.3 $\pm$ 0.3	1.2 $\pm$ 0.4	1.4 $\pm$ 0.4	1.5 $\pm$ 0.4	1.4 $\pm$ 0.5	1.4 $\pm$ 0.6
BN-rats	59	1.4 $\pm$ 0.4	1.6 $\pm$ 0.6	1.7 $\pm$ 0.5	1.3 $\pm$ 0.4	1.3 $\pm$ 0.5	0.9 $\pm$ 0.2
Male	65	1.4 $\pm$ 0.4	1.3 $\pm$ 0.5	1.6 $\pm$ 0.5	1.4 $\pm$ 0.4	1.3 $\pm$ 0.5	1.1 $\pm$ 0.4
Female	61	1.3 $\pm$ 0.4	1.4 $\pm$ 0.5	1.5 $\pm$ 0.5	1.3 $\pm$ 0.4	1.4 $\pm$ 0.5	1.2 $\pm$ 0.6
Starved	62	1.3 $\pm$ 0.4	1.4 $\pm$ 0.6	1.5 $\pm$ 0.4	1.3 $\pm$ 0.4	1.4 $\pm$ 0.5	1.1 $\pm$ 0.5
Non-starved	64	1.4 $\pm$ 0.4	1.3 $\pm$ 0.5	1.5 $\pm$ 0.5	1.5 $\pm$ 0.4	1.3 $\pm$ 0.5	1.2 $\pm$ 0.5
Young (1-4 months)	45	1.4 $\pm$ 0.4	1.3 $\pm$ 0.4	1.5 $\pm$ 0.5	1.4 $\pm$ 0.4	1.4 $\pm$ 0.5	1.0 $\pm$ 0.3
Medium (5-8 months)	42	1.3 $\pm$ 0.4	1.5 $\pm$ 0.6	1.6 $\pm$ 0.5	1.3 $\pm$ 0.4	1.4 $\pm$ 0.5	1.2 $\pm$ 0.6
Old (> 8 months)	39	1.3 $\pm$ 0.3	1.4 $\pm$ 0.5	1.6 $\pm$ 0.5	1.4 $\pm$ 0.4	1.4 $\pm$ 0.5	1.2 $\pm$ 0.5

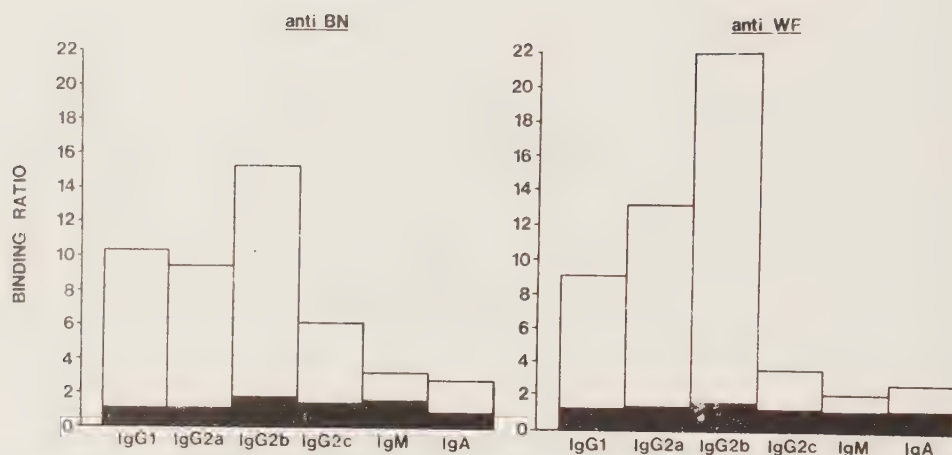


Fig. 4. Binding of alloantisera to allogeneic (open bars) and syngeneic (shaded bars) target cells in a criss-cross manner. The binding is expressed as the ratio between bound radioactivity for alloantiserum and NRS.

Ig-binding levels in serum from untreated rats

The Ig levels of sera from untreated rats are shown to be normally distributed by the use of the Kolmogorov-Smirnov one sample test (Siegel, 1956) and to have homogeneous variances by the use of the Bartlett's test (Winer, 1971). Since IgG2a and IgA showed normal distribution only after logarithmic transformation, all calculations were done on the logarithms of the binding ratios. The means and standard deviations shown in Table I are values recalculated after that sera with ratios higher than mean + 3 S.D. have been excluded from the material. As Table I shows, no major differences were found between the various groups of rats. However, analysis of covariance (Winer, 1971) showed that WF rats had a statistically significantly lower level of IgG2a ($F(1,114) = 13.6$; $P < 0.001$) and a higher level of IgA ($F(1,114) = 46.9$; $P < 0.001$) compared with BN rats. The percentage of false positives is shown in Table II. The level for positive response is set to 2.0 (mean + S.D.) or 2.5 (mean + 2 S.D.). This table includes all sera.

TABLE II
PERCENTAGE FALSE POSITIVES IN UNTREATED RATS

	IgG1	IgG2a	IgG2b	IgG2c	IgM	IgA
Limit 2.5 ^a	1.6	8.7	8.6	1.6	6.3	5.5
Limit 2.0 ^b	5.5	14.2	18.8	10.1	12.6	11.0

^a Limit 2.5 = mean + 2 S.D.

^b Limit 2.0 = mean + S.D.

Discussion

We describe the production and characterization of rabbit antisera against the classes and subclasses of rat immunoglobulins and their use in a ^{125}I -protein A-binding assay for detection and quantitation of rat antibodies binding to antigens on the cell surface. To obtain antisera against rat IgG subclasses rabbits were immunized with preparations from a normal serum pool. The use of such preparations was judged to be preferable compared to the use of monoclonal or myeloma immunoglobulins, as the risk of obtaining anti-idiotypic reactivities is diminished and as it gives an equal reactivity against the whole subclass population.

For IgM and IgA monoclonal immunoglobulins from hybridoma cultures had to be used. Pure IgA and IgM preparations could not be made from normal rat serum and antisera obtained after immunization with the impure preparations showed reactivity mainly with IgG. This may also indicate that the rat IgM and IgA are less immunogenic in rabbits than IgG, as it has been reported by others (Goosen et al., 1981) that human immunoglobulins vary in immunogenicity in rabbits. Immunizations with monoclonal IgM and IgA at much higher concentrations and purity of antigen resulted in antisera reactive with the heavy chains of these classes. Absorptions had to be performed as reactivities against common determinants on light or heavy chains were also detected.

When testing the specificity of the antisera in the indirect protein A-binding assay using solid-phase monoclonal immunoglobulins, cross-reactivities were detected which could not be detected in double immunodiffusion. Additional absorptions had to be performed before the antisera showed monospecificity at concentrations of solid-phase immunoglobulins at least 10 times higher than those detected as cell surface-bound antibodies from test serum. As solid-phase immunoglobulins we preferred to use monoclonal immunoglobulins of various (sub)classes obtained from tissue culture to avoid the contaminating immunoglobulins present in ascitic fluid of animals with growing hybridomas. Monoclonal immunoglobulins offer the advantage of purity by definition, as compared with IgG subclasses fractionated from serum. This enabled us to differentiate between true cross-reactivity of an antiserum and contamination of the target antigen preparation. Testing of specificity of antisera against IgM and IgA were performed with solid-phase monoclonal immunoglobulins obtained from hybridomas different from those used for immunization so as to avoid testing for reactivities against idiotypic determinants.

It is a great advantage to use radiolabeled protein A in this binding assay since it allows labeling to high specific activity, purity and high affinity for rabbit IgG (Goding, 1976, 1978). Protein A has been shown not to bind to rat Ig or to bind with low affinity (IgG1 and IgG2c (Nilsson et al., 1982)). This low protein A binding to rat antibodies, simplifies the (sub)class analysis as the background is low for (sub)classes, not detected by the intermediate step of the rabbit antiserum. It has been previously demonstrated (Brown et al., 1977) that the use of labeled SpA instead of labeled anti-immunoglobulin increases the sensitivity of the test. It is also easier to keep one reagent radiolabeled than 6 different reagents, which in addition would require Ig fractionation before labeling. This advantage of selective analysis

may be achieved also in those species whose IgG have a higher protein A-binding capacity by introducing a parallel control of normal rabbit serum instead of rabbit antiserum to give an estimate of the direct protein A binding to the primary antibodies.

The antibody binding was shown to be linear over a wide range of concentrations (Fig. 2). One implication of this is that serum can be tested at a single dilution without any loss of information. No competition of one (sub)class of immunoglobulin with another could be demonstrated even in high concentrations of positive serum, indicating that the antigen is in large excess. The saturation experiment (Fig. 3) also demonstrates that it is possible to analyze quantitatively the primary (rat) antibody and that the results are independent of the second and third reagents. The sensitivity is high, making it possible to detect about 1 ng of bound antibody. It should however be noticed that the amount of antibody bound depends on the product of concentration and avidity of this antibody.

The degree of binding of Ig from normal rat serum and the frequency of falsely positive normal rats were determined by assaying sera of 126 normal rats of various ages, and of different sexes, strains and nutrition statuses. The binding levels of the various immunoglobulins were normally distributed and no major differences were seen between the various groups of rats. The immunological importance of the statistically significant difference between WF and BN rats in the analyses of IgG2a and IgA could not be evaluated in this study. The limit set for positive response will depend on the aims of the analysis where this binding assay technique is to be used. For some analyses a lower limit will be selected, because it is important not to miss any positive response (i.e., the frequency of false negatives must be low) even if this will give a higher number of false positives. For another analysis it may be important to keep the false positives as low as possible and a higher level has to be used. It is also important to consider the specificity of the reactions. Some of the reactions seen as false positives may be true antibody reactivities against an antigen in the test system and the difference between 'false' and 'true' positives is then quantitative rather than qualitative.

The developed binding assay allows convenient analysis of 6 different classes or subclasses of rat immunoglobulins without separation or treatment of the test serum. The assay will be further adapted for analysis of tumor-specific antibodies in rats and used for studies of the humoral immune response against rat colon carcinoma. It will also be used in the screening and characterization of monoclonal antibodies produced by the hybridoma technique.

Antigen targets other than living cells may also be used in the assay, e.g., fixed cells, cell preparations or purified antigens adsorbed to the bottom of the plate.

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Antigen-Independent Binding of Rat Immunoglobulins in a Radioimmunoassay. Solutions to an Unusual Background Problem

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A high non-specific binding of immunoglobulins to plastic surfaces was noted with a number of rat sera, when tested in an indirect ¹²⁵I-labeled protein-A assay for detection of cell-surface-bound rat immunoglobulins of various classes and IgG subclasses. This type of non-specific binding was found with all types of Ig. The degree of binding varied with the type of test plate used and fluctuated with time among sera drawn sequentially from the same donors. Coating test wells with fetal calf serum supplemented with BSA, gelatin or fibrinogen did not eliminate the reactions. The immunoglobulins bind directly to the polystyrene, and not to antigens present in fetal calf serum or autoantigens in rat serum.

Two different approaches were used to eliminate the nonspecific reaction. When living cells were used as target antigens, exclusively cell-bound radioactivity was eluted with the non-ionic detergent Nonidet P40, which solubilizes the cell membrane without breaking the protein-A/rabbit IgG, rabbit IgG/rat Ig, or rat Ig/plastic interactions. When rat serum antibodies are tested on target antigens adsorbed on non-tissue culture grade plates, non-specific binding may be avoided by including 0.05% Tween 20 in the incubation mixture.

Key words: *binding test – rat Ig (sub)classes – cell surface antigens – plastic binding of Ig – non-specific Ig binding in RIA*

Introduction

A variety of radioimmunoassay (RIA) techniques exists for assay of antibodies to cell surface antigens on intact living cells. When reactivity to soluble antigens is analyzed, the target antigens are adsorbed or coupled to a solid phase in order to simplify the separation of bound and free reagents. Several of these techniques make use of the 96-well microtest plate system, which is optimal for handling of large numbers of samples. The use of this system does, however, introduce a new problem in the form of non-specific binding of sample or reagents to reactive sites on the

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surface of the plastic (usually polystyrene). The traditional way of eliminating this binding is to include a non-reactive protein or detergent in the incubation solutions or to precoat the test wells with proteins to saturate the reactive sites. The problems solved by these precautions mostly arise from general, nonspecific reactivity, i.e. background occurring to the same degree in controls and in most of the test samples.

Sequential sera from rats with chemically induced primary colon carcinomas have been screened for antitumor antibodies by use of a previously described RIA technique for quantitative analysis of rat Ig(sub)classes binding to surface antigens of tumor cells (Nilsson et al., 1982). During analysis of the specificity of positive sera, it was noted that some sera showed the same binding pattern when tested on a variety of cells and even when cells were excluded. The appearance of this non-specific binding capacity was not dependent on tumor growth or chemical treatment, since it was found also in untreated rats and in carcinogen-treated rats which remained tumor-free.

These non-specific reactions were unusual and different from previously described background reactivities, since they were detected in a test system with precoated plates (20% FCS) and with test serum supplemented with a high protein concentration (20% FCS) during incubation. Neither further coating with proteins nor addition of detergent was effective in eliminating the reaction. The binding varied with the type of plate used, and it appeared that the binding did not reflect a true immunochemical interaction.

We describe here two different approaches to eliminating this non-specific binding. When adherent living cells grown in tissue-culture plates were used as target antigens, we investigated whether a detergent may be selected which elutes the cell-bound radioactivity but does not affect the activity bound to plastic. When soluble proteins were used as target antigens, we investigated whether a type of plastic plate may be selected which allows elimination of the problem by addition of detergent to the test sera.

Materials and Methods

Animals, serum and cells

Rats of the strains Wistar/Furth (WF) and Brown Norway (BN) were used. They were maintained by continuous, single-line, brother to sister mating. F₁ hybrids (WB) of BN male and WF females were also used. The rats were kept on a standard pellet diet and water ad libitum.

A tissue culture cell line of a colon adenocarcinoma (DMH-W49) of WF origin (Nilsson et al., 1982) was passaged serially in Waymouth's medium supplemented with 20% fetal calf serum (FCS) and 4 mM L-glutamine (W20-medium). The cells were regularly checked for contamination with mycoplasma and other contaminants by fixation and staining for DNA with the fluorescent dye Hoechst 33258 as described by Chen (1977). Rats were bled under ether anesthesia by cardiac puncture (for serum pools) or by capillary drainage from the retro-orbital sinus (for individual sera). Sera were separated and stored at -20°C .

Radioimmunoassay of rat antibodies to cellular and adsorbed antigens

Quantitation of antigen-binding rat antibodies of various immunoglobulin (sub)classes was performed by the technique described previously (Nilsson et al., 1982). When viable cells were used as targets in the test, tissue culture grade sterile 96-well flat-bottomed microtiter plates from various sources were used. Falcon Microtest II plates (No. 3042; Falcon Products Oxhard), Costar TC-cluster (No. 3596, Costar, Cambridge MA), Nuncclon Delta (Nunc Roskilde), Titertek TC-plate (Flow No. 76-004-05) and Dynatech M29 ART (Flow Laboratories) were all of the rigid polystyrene type. Non-sterile polystyrene plates not intended for tissue culture (Titertek, Flow No. 76-301-05) or tissue culture grade plates were coated with the proteins used as solid phase antigens in the RIA-technique. The proteins were diluted in phosphate buffered saline (PBS, 10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.3) to a concentration of 5 µg/ml (or as specified) and 50 µl per well were incubated for 3 h at 37°C. After 2 washes with 200 µl PBS, the protein-reactive sites on the plastic were blocked by overnight incubation at 37°C with 200 µl W20-medium. The protein concentration in the fetal calf serum used was about 25 mg/ml. After incubation with rat sera for 45 min, the plates were washed and incubated with a second antibody, rabbit anti-rat Ig (sub)class serum. After another wash protein-A (SpA) labeled with ¹²⁵I (Bolton and Hunter, 1973; Langone, 1980) was added.

Adsorption of rat serum on micro-columns

Micro-columns (5 × 25 mm, 0.5 ml) with proteins covalently coupled to Sepharose CL4B (Pharmacia Fine Chemicals) by the cyanogen bromide technique (Porath et al., 1967) were used for adsorption of reactive rat sera. One ml FCS and 0.5 ml NRS were coupled per ml of Sepharose for adsorption on FCS and NRS, respectively. Octyl- and phenyl-Sepharose CL 4B were obtained from Pharmacia Fine Chemicals. Sonicated mycoplasmal organisms from about 7 ml of 3 day suspension culture of a strain isolated from an infected rat cell culture in our laboratory were coupled per ml activated Sepharose (M-Ag). Non-polar polymeric adsorbents of polystyrene-divinylbenzene (Bio-Beads SM-2) were obtained from BioRad Laboratories and used either untreated or precoated with 1 vol. undiluted NRS overnight at room temperature.

The columns were washed with complete Waymouth's medium without FCS (WO-medium) before 20 µl undiluted rat serum were applied. After about 10 min incubation the columns were eluted with 1.6 ml WO-medium at a rate of about 10 ml/h. The eluates were collected in tubes containing 0.4 ml FCS. Unadsorbed serum and serum adsorbed on Sepharose without coupled proteins were used as control.

Experiments with various detergents

In order to decrease background binding, detergents were used in 2 different ways. First, detergent was added to the rat serum during the first incubation step of the assay, to inhibit binding to plastic dependent on hydrophobic interaction. For this purpose Tween 20 (KEBO) at concentrations of 0.01–3.2% (v/v) was tested.

The second approach was to seek detergents that would elute cell-bound radioactivity exclusively by solubilizing the cells without splitting the antibody-antigen or antibody-protein-A interactions, as is the case when SDS or low pH buffer is used. Sodium dodecyl sulphate (SDS, BDH), Nonidet P-40 (NP-40, Shell), Triton X-100 (Sigma T-6878), Tween 20 (KEBO, Stockholm), Tween 40, Tween 80, Tween 85 (ICI, Gothenbourg) and Brij 35 (ICI) were added to PBS at a concentration of 10 g/l. Hexadecyltrimethylammonium bromide (Cetrimide; Sigma H-5882) was dissolved to 100 g/l in PBS supplemented with 2 mM EDTA and 0.14 M NaCl. Sodium deoxycholate (DOC, Merck-6504) was dissolved to 10 g/l in 10 mM Tris/HCl, pH 8.1. The detergents were used in the elution step after ^{125}I -labeled SpA incubation without other modification of the assay. Cell disruption properties were analysed with allo-antiserum binding to cells and the effect on antibody-antigen and antibody-SpA reactions were evaluated in a cell-free system.

Results

A strong unusual type of background reactivity was detected in a proportion of various types of rat sera. Sera of 128 normal, untreated rats were assayed for Ig-binding to living DMH-W49 colon cancer cells by use of the standard technique (Nilsson et al., 1982), with SDS elution of the content of the wells as the final step. Less than 10% had a binding ratio higher than 2.5 for the different immunoglobulin types. When the limit was set to 2.0 the number of positives was high, and with the exception of IgG1 was higher than 10% (Table I). Sera of rats treated with DMH with or without tumor development often showed reactivity.

In order to inhibit non-specific binding, precoating with various proteins was attempted. This was not, however, very successful with plates of tissue culture grade. Although the binding of some sera was slightly decreased (10–20%), it was not possible to eliminate completely the reactivity of any of the sera by coating the plates with BSA, gelatin or fibrinogen in combination with W20-medium. In some

TABLE I

PERCENTAGE OF FALSE POSITIVES IN SERA FROM UNTREATED RATS TESTED ON DMH-W49 CELLS

Detergent ^a	Limit ^b	IgG1	IgG2a	IgG2b	IgG2c	IgM	IgA
SDS	2.0	5.5	14.2	18.8	10.1	12.6	11.0
	2.5	1.6	8.7	8.6	1.6	6.3	5.5
NP-40	2.0	0	0	2.3	0	0.8	0
	2.5	0	0	1.6	0	0	0

^a Type of detergent used in the assay for final solubilization of cells.

^b Mean binding ratio + S.D. (= limit 2.0) and mean binding ratio + 2 S.D. (= limit 2.5) used to define positive sera.

experiments, Tween 20 was used to supplement the diluted rat sera to eliminate hydrophobic interactions in plates precoated with W20-medium. Tween 20 did not decrease the binding. Although the reactivity of some sera was reduced to some extent by adsorption on FCS-, NRS- or M-Ag Sepharose, true immune reactions did not seem to be responsible for the binding. Octyl- and phenyl-Sepharose and SM-2 Bio-Beads were used to adsorb reactivity associated with hydrophobic components. As with Tween 20, these adsorptions did not decrease reactivity, indicating that binding of immunoglobulins to the plastic was not due to hydrophobic interactions.

The experiments described above were all carried out in plates of tissue culture grade. When comparing plates from various commercial sources, it was noticed that the various types of tissue culture grade plates were very similar with regard to this type of immunoglobulin binding. However, the behavior of non-tissue culture grade plates was quite different. For the same serum, background reactivity of different immunoglobulin types could be detected depending on the type of plate used (Fig. 1). For example, a serum might show a positive IgG2a response on tissue culture grade plates, but IgM binding on non-tissue culture grade plates (serum A, Fig. 1). No general correlation was, however, observed between immunoglobulin type and

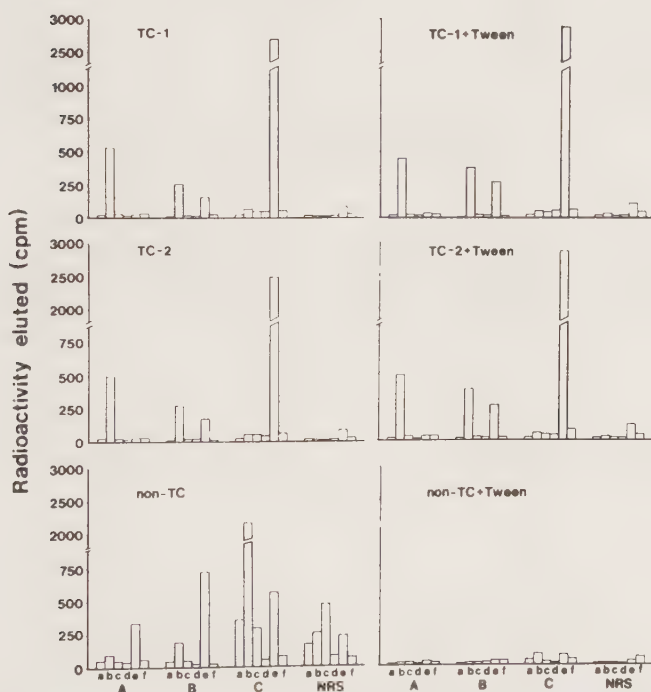


Fig. 1. Non-specific binding of rat Ig to FCS-coated plates of tissue-culture grade (TC-1, TC-2) and non-tissue-culture grade (non-TC) with and without addition of 0.05% Tween 20 to the rat sera during incubation. Sera containing different reactive immunoglobulins (A-C) and normal reference serum (NRS) were tested. a = IgG1, b = IgG2a, c = IgG2b, d = IgG2c, e = IgM, f = IgA. Bound radioactivity was eluted with 1% SDS.

plate type. The binding to non-tissue culture grade plates appeared to be of hydrophobic type, since with all sera tested the reactivity was completely abolished when 0.05% Tween 20 was included in the incubation step with rat serum (Fig. 1).

When adherent cells are used as targets in the RIA, plates of tissue-culture grade are required for most cell types. Various detergents were screened to find one with adequate cell-solubilizing properties which did not disturb the rat Ig/plastic, rat Ig/rabbit IgG, or the rabbit IgG/SpA bonds. Fig. 2 shows that NP-40 and Triton X-100 fulfilled these criteria very well, while the other detergents were either poor solubilizers or interfered with some of the interactions mentioned. NP-40 was selected for further studies. Fig. 3 shows the results with sequential sera from an untreated rat with high non-specific reactivity, as seen with SDS elution (Fig. 3A). When NP-40 was used for elution no non-specific reactivity was detected (Fig. 3B). The frequent false positive reactions recorded when sera of 128 normal rats were assayed with SDS elution as the final step were almost entirely eliminated if NP-40

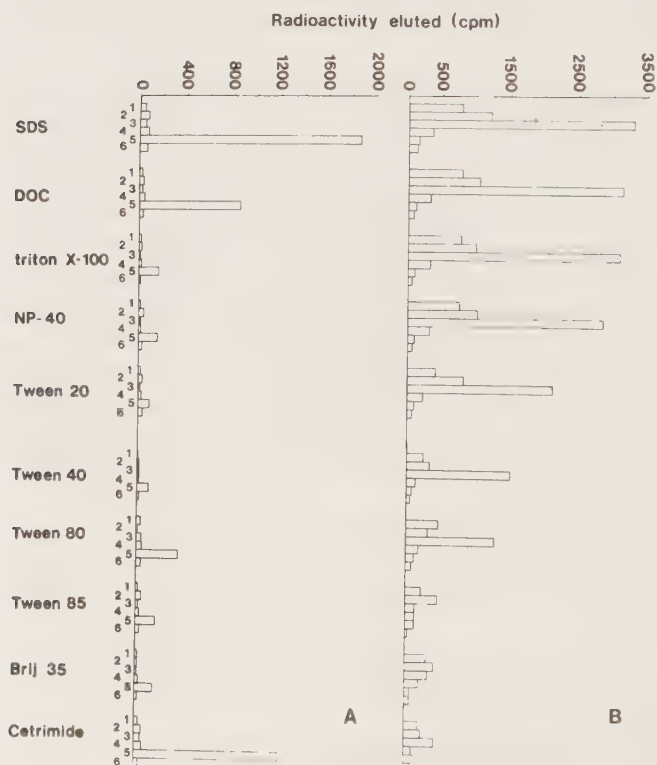


Fig. 2. Elution of bound radioactivity by the use of various detergents. A: Serum showing non-specific binding tested in FCS-coated wells. B: Anti-WF serum tested on living DMH-W49 colon cancer cells. In (A) the radioactivity eluted with Triton-X-100, NP-40, Tween 20, and BRIJ 35 is dramatically reduced compared with SDS (i.e. binding ratio < 2.0). Plates of tissue culture grade were used in both (A) and (B). 1 = IgG1, 2 = IgG2a, 3 = IgG2b, 4 = IgG2c, 5 = IgM, 6 = IgA.

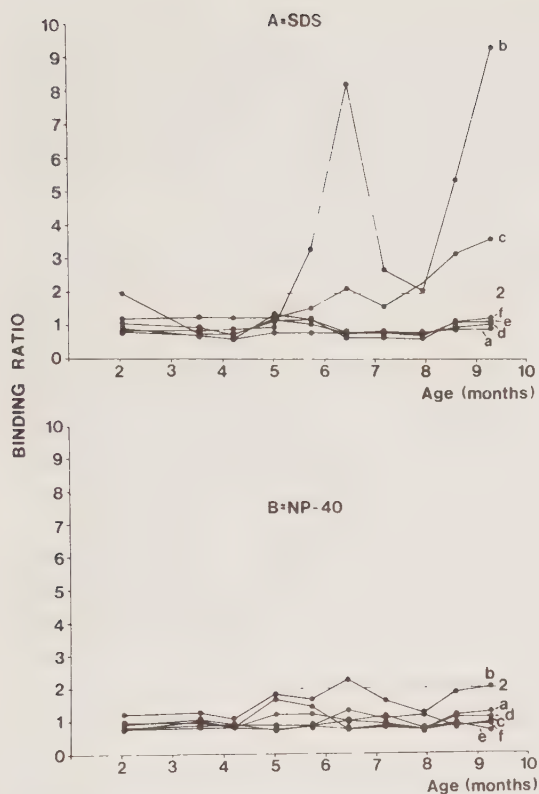


Fig. 3. Binding reactivities of rat immunoglobulin in sequential sera from an untreated BN rat to wells with living DMH-W49 colon cancer cells in tissue culture grade plates. Elution of bound radioactivity was with 1% SDS (A) or 1% NP-40 (B). The binding patterns were similar when the cells were excluded (not shown). All sera were run in the same test. a = IgG1, b = IgG2a, c = IgG2b, d = IgG2c, e = IgM, f = IgA.

was used instead of SDS for elution of bound radioactivity (Table I). Only 4 rats out of 128 had a binding ratio > 2.0 , considering all immunoglobulin types together.

Discussion

The RIA technique for quantitative analysis of the binding of rat Ig(sub)classes to cell-surface antigens previously described (Nilsson et al., 1982) was used for screening tumor-binding antibodies in sera of DMH-treated rats. When the specificity of the reactions was evaluated, however, some sera showed the same binding pattern independently of the cell type used. The same results were obtained when sera were assayed in wells from which all target cells had been excluded. This binding pattern was also seen with sera from untreated rats. This background binding occurred in all immunoglobulin (sub)classes.

The background reactivities of normal rat serum pools are acceptable (i.e. about 100 cpm at dilution 1/100). The high reactivities of some individual sera are, therefore, both qualitatively and quantitatively of a different nature. The reactivity of serum from a given rat may be low for a long time, then increase and remain high for some months, thereafter decreasing to normal levels again (Fig. 3A). As also shown in earlier studies (Nilsson et al., 1982) these reactivities are not age dependent.

The traditional way of preventing non-specific background binding in radioimmunoassays is to block the reactive sites on the plastic with various proteins. Usually, bovine serum albumin (BSA) (Tsu and Herzenberg, 1980) or gelatin are used. Coating with gelatin has been reported to eliminate most non-specific binding of monoclonal Ig to plastic in plates of tissue-culture grade (Lansdorp et al., 1980). Combined pretreatment of the plates with FCS and Tween 20 has also been reported satisfactory (Douillard et al., 1980). Fibrinogen is unique in its ability to bind to plastic and can displace previously bound proteins (Parsons, 1981). When we tested these coating procedures, however, none of them prevented to any great extent the binding found with our rat sera.

Binding of rat serum immunoglobulins might have reflected a true immunochemical interaction with proteins present in fetal calf serum, as has been shown to be the case with several human sera (Liao et al., 1979). Also to be considered is the possible presence of autoantibodies against Ig or other rat serum proteins and binding of such antibodies to rat proteins adsorbed to the plastic. This seems unlikely in view of the failure of adsorptions of reactive sera on FCS- or NRS-columns to decrease such reactivity.

In a similar test system, Matzku and Zöller (1979) described analyses of rat sera obtained from rats carrying tumor transplants. These sera showed almost identical binding patterns when tested on tumor cells and normal cells, respectively. No tests were reported, however, with FCS-coated plates without target cells. It was concluded that this nonspecific binding derived from cross-reactivities with mycoplasma, and that the only way to avoid it was to use SPF-rats. The non-specific reactions in our sera did not significantly change after adsorption on mycoplasmal antigens from a strain isolated from an infected rat cell culture in our laboratory.

The background phenomena are related to the type of plates used. In non-tissue-culture grade plates the interaction seems to be hydrophobic as it can be completely eliminated by addition of Tween 20 during the incubation step with rat serum. Tween 20 had, however, no effect on binding to various plates of tissue-culture grade, so that these interactions appear to be hydrophilic. The usual way to inhibit such interactions is to increase the ionic strength, but this is not compatible with the use of adherent living cells.

In order to elute only cell-bound radioactivity, various detergents were tested. Nonidet P40 was shown to solubilize the cell membranes without splitting protein-A/rabbit IgG, rabbit IgG/rat Ig, or rat Ig/plastic interactions. It has been previously reported that non-ionic detergents in a concentration up to 2% do not influence antigen-antibody interactions (Crompton and Parkhouse, 1972). With NP-40, the frequency of false positives was dramatically diminished. This illustrates that non-specific binding to the cell surface is rare.

In conclusion, when cells are used as targets exclusive elution of cell-bound radioactivity is achieved with 1% NP-40. When the NP-40 supernatants have been harvested the radioactivity bound to plastic may be eluted with 1% SDS to provide information about background reactivity. For analysis of antibodies against soluble proteins adsorbed to non-tissue culture grade plates, Tween 20 should be added during the incubation step with the test serum. At the end of the test, radioactivity is eluted with low pH buffer or SDS. Non-adherent target cells attached via Con-A to the bottom of wells (Moolten and Schreiber, 1980) may be used in either type of plate provided that NP-40 is used for elution (data not shown).

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USE OF BUTANOL-EXTRACTED ANTIGENS OF RAT COLON CARCINOMA
FOR ANALYSIS OF HUMORAL TUMOR IMMUNITY.

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running title: Humoral immunity to rat colon carcinoma

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SUMMARY

It is demonstrated that soluble tumor associated antigens, extracted from in vivo grown tumors using a single phase solution of 2.5 % 1-butanol, can be used in an indirect ^{125}I -SpA radioimmunoassay for detection of humoral antibodies to syngeneic rat colon carcinomas. The optimal conditions for preparation and use of the butanol tissue extracts as target antigens are described. The Ig(sub)classes of the bound antibodies are determined. Two types of specificity tests for antibodies binding to tumor extracts are established. One is competitive binding inhibition of sera by butanol extracted tumor antigens. The other test involves immunosorption of sera on sepharose columns with covalently linked extracts of colon carcinomas and other neoplastic or normal tissues prior to testing in the binding test. With these techniques tumor-reactive antibodies are detected in sera of rats immunized to tumor isografts. The specificity analysis shows that these antibodies are directed to tumor associated antigens shared by some DMH-induced rat colon carcinomas but not expressed by normal tissues.

INTRODUCTION.

Cells of many neoplasms express surface molecules which distinguish them from their normal counterparts. Chemically induced tumors frequently display unique antigens capable of eliciting a specific host immune response leading either to rejection or to growth enhancement of the tumor. Although antibodies to antigens of some tumors of this type have been known for some time, several problems have prevented the precise determination of their specificity and functions. Most assays have been dependent on tissue cultured cells as the source of target antigens and have often depended on a functional activity of the antibodies. Consequently they have detected only a subpopulation of the antibodies. Tissue culture cell lines are susceptible to a variety of artifacts including selection of clones during explantation, in vitro transformation, infection by mycoplasma or other contaminants. For some tumor lines variation in the expression of cell surface antigens during the cell cycle have been reported (3). There are also difficulties to explant and maintain cell lines from normal tissues for tests of specificity. In order to avoid several of these problems attempts have been made to use solubilized molecules from cell membranes instead of living cells. Cell surface molecules may be solubilized from whole cells or from purified cell membranes by a variety of techniques including the use of detergents (1), 3 M KCl (12), 4 M urea (5) or enzyme treatment (14). Recently, a single-

phase solution of 1-butanol was used to obtain a fraction of tumor specific transplantation antigens from chemically induced murine fibrosarcomas (7).

We describe here the establishment of a radioimmuno assay for antibodies to antigens associated with rat colon carcinoma, using 1-butanol tissue extracts as target antigens. By use of developed specificity tests, those antibodies were shown to be directed against tumor associated antigens shared by DMH-induced rat colon carcinomas.

MATERIALS AND METHODS:

Animals and tumors

Rats of the strains Wistar/Furth (WF) and Brown Norway (BN) were used as tumor and serum donors. They were maintained by continuous, single-line brother to sister mating. The rats were kept on a standard pellet diet and water ad libitum. Nude (Rowett rnu/rnu) rats (13) were used to obtain tumor materials for some of the antigen preparations.

Colon adenocarcinomas (W49, W163, W6038, BN7014) induced with 1,2-dimethylhydrazine (DMH) were kept in serial intraperitoneal passage in syngeneic rats. A kidney carcinoma (WK 2027) and a T-lymphoma (W 62) appeared in DMH-treated rats which did not develop other types of tumor. Tissues of some tumors were obtained after a single passage to nude rats.

Sources of rat serum

Sera were obtained from tumor-bearing rats and from rats immunized in various ways against the tumors in question. Immunization techniques will be reported elsewhere. Alloantisera were raised in rats by three biweekly intraperitoneal injections of 2×10^7 allogeneic spleen cells. Serum was collected one week after the last injection. Pooled sera of normal, untreated inbred WF female and male rats of various ages were used as normal reference serum (NRS) in the assays.

Rats were bled under ether anesthesia by cardiac puncture (for serum pools) or by capillary drainage from the retro-orbital sinus (for individual sera). Sera were separated and stored at -20 C.

Antigen extraction

Extraction with 1-butanol was performed by a modification of the method of LeGrue et al (7). Tumor tissue and control tissue were processed in an identical way and besides the butanol step the whole procedure was performed at 4C. The tissues were excised from the rats and immediately cut into small pieces by a pair of scissors and then pressed through 60-mesh stainless steel screens into 50 mm petri dishes containing PBS (calcium- and magnesium- free 10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.3). The tissue fragments were washed four times with about ten volumes of PBS before it was suspended in ten volumes of 2.5 % (v/v) 1-butanol (Merck) in PBS. The suspension was incubated for 5 min in room temperature and was then centrifuged at 500xg for 10 min. The supernatant was dialyzed against 100 volumes of PBS with two changes in 48 hours followed by centrifugation at 40 000xg for 60 min (Sorwall SS-34). The resultant supernatant was collected and stored at -80 C and is designated as crude butanol extract (CBE).

An alternative method was used for extraction of cell membrane preparations. One hundred grams of frozen tissue were thawed in 200 ml of 250 mM sucrose, 5 mM $MgCl_2$, 25 mM KCL, 2 mM PMSF (phenylmethyl-sulfonyl

fluoride) and 50 mM TRIS, pH 7.3, and were homogenized in an Omnimixer (Sorvall, half max setting for 1 min). The homogenate was centrifuged at 1000xg for 10 min and the resultant supernatant was centrifuged at 100 000xg for 60 min. The crude membrane pellet was washed once in PBS and was resuspended in 10 volumes of 2.5 % 1-butanol in PBS and incubated for 5 min at room temperature. The membrane preparation was centrifuged at 100 000xg for 60 min and the supernatant was dialyzed against PBS followed by centrifugation at 40 000xg for 60 min to remove nonsoluble material. The extracts have been stored for up to seven months without detectable decrease in reactivity. Protein concentrations were determined by the use of the Coomassie Brilliant blue technique (BioRad Laboratories; (2)) using Ovalbumin as a standard.

Radioimmunoassay of rat antibodies to CBE

Quantitation of CBE-binding rat antibodies of various immunoglobulin (sub)classes was performed by a modification of a previously described technique (10, 11). The assay was performed in 8-well flat-bottomed stripes (Dynatech Immulon removastripes no M1798A ;Laboratorie Design AB, Stockholm, Sweden) placed in 96-well holders (Dynatech M73A) to receive the standard 96-well microtest plate format. Crude butanol extracts were diluted in PBS to a concentration of 10 - 20 µg/ml (or as specified) and 50 µl per well were incubated for 18 h at 4 °C. After two washes with 200 µl PBS, the protein-

reactive sites on the plastic surface were blocked by incubation for one hour at room temperature (RT) with 200 μ l coating/dilution buffer (CD-buffer; 20 % fetal calf serum, 10 mM sodium phosphate, 10 mM HEPES, 0.15 M sodium chloride, 5 mM sodium azide pH 7.3). The CD-buffer was aspirated and 50 μ l rat serum diluted in CD-buffer were added. After incubation for one hour at RT, the sera were aspirated and the plates were washed twice with 200 μ l washing solution (WB-buffer; 10 mg/ml BSA (Sigma A-4503), 10 mM sodium phosphate, 10 mM HEPES, 0.15 M sodium chloride, 5 mM sodium azide, pH 7.3). Rabbit antisera to rat Ig(sub)classes were added in volumes of 50 μ l diluted in CD-buffer and the plates were again incubated for one hour at RT and washed twice with 200 μ l WB-buffer. A volume of 50 μ l of protein-A (SpA), labelled with 125 I by use of the Bolton-Hunter method (6) and diluted in CD-buffer was added and incubated for one hour at RT. After two washes the bound radioactivity of the individual wells was counted in a gamma counter (Auto-gamma 500C; Packard Instruments) for two minutes per well. Each sample was run in duplicate and always tested for binding to tumor and control extracts.

Specificity analysis of CBE-binding rat serum

Two different approaches, competitive binding inhibition and solid phase immunosorption, were used for evaluation of the binding specificity of reactive rat sera. In experiments with competitive binding inhibition 50 μ l of various dilutions of CBE and 50 μ l of diluted rat serum

were simultaneously added to wells with preadsorbed extracts. After mixing the plate was incubated for two hours at RT. The mixture was aspirated and bound rat Ig quantitated in a radioimmunoassay.

Solid phase immunosorption was performed on micro-columns with covalently linked butanol extracts. Activated CH-sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen and washed with 1 mM HCl followed by washes with PBS. One ml of extract (corresponding to 0.1 ml tissue) was added per ml sepharose and was incubated under end over end mixing for 18 h at 4 °C. The gels were washed and blocked with 1M ethanolamine, pH 8.0, for three hours at 4 °C. After four washing cycles with 0.1 M NaHCO₃, 0.5 M NaCl pH 8.3 and 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0, the gels were ready to use. Activated CH-sepharose treated in the same way but without protein added was used as control gel (blank-sepharose).

For immunosorption micro-columns (5x20 mm, 0.4 ml) were packed and equilibrated with CD-buffer before 50 µl undiluted rat serum were applied. After incubation for 10 min the columns were washed with one ml CD-buffer at about 3 ml/h. The effluents were collected and analyzed in the radioimmunoassay for binding to CBE. The whole procedure was performed at room temperature. The columns could be reused after elution with 0.1 M glycine/HCl pH 3.0 buffer or with 3 M NaSCN in PBS.

RESULTS

The 1-butanol extraction procedure, described by LeGrue et al (7), was modified by exclusion of the trypsinization of the tissues before the extraction. The volume of 2.5 % 1-butanol used was not critical as extraction with one volume of 1-butanol resulted in the same antigenic reactivity per amount of protein as ten volumes of 1-butanol, when used as target antigen in the radioimmunoassay. However, extraction with ten volumes resulted in a higher yield of antigen (5 mg total protein/ ml tissue pellet) than when one volume was used (2 mg total protein/ ml tissue pellet). This procedure has also been adapted to the use of crude cell membrane preparations as material for extraction (data not shown). When tissues were obtained from WF or BN rats, the extracts contained detectable amounts of immunoglobulins. Passage of tumors once in nude rats did not result in any improvement, i.e neither lower immunoglobulin content nor higher antigenicity per amount of protein.

Various adsorption conditions were investigated to establish the optimal procedure for the use of CBE as target antigen in the radioimmunoassay. The Immulon plastic (Dynatech M129A, M1798A) was found to be superior to untreated polystyrene (Titertek, Flow 76-301-05) or the tissue culture treated polystyrene (Dynatech M29 ART) and untreated polyvinyl Chloride (Costar 2596).

Titration of the extracts showed that the binding

reactivities reach a plateau at concentrations higher than 20 $\mu\text{g/ml}$, but no decrease in reactivities was observed with concentrations up to 500 $\mu\text{g/ml}$ (chart 1). The pH (4-10) and the ion strength (0-4 M NaCl) of the adsorption buffer did not drastically influence the adsorption (chart 2A, 2B). In tests of the influence of butanol on adsorption it was shown that concentrations of 1-butanol up to 5 % (v/v) in PBS did not inhibit the reaction (chart 2C). The duration and temperature are important. Incubation for 18 h at 4 C resulted in 2 - 3 times higher reactivity than incubation for 3 h at 37 °C. The subsequent steps of the radioimmunoassay are mainly performed as previously described (10), but incubations are performed for one hour at RT instead of 45 min at 37 °C. The reproducibility of the test was good, with an intertest variation of 5 % as determined in four tests during a one month period.

With this radioimmunoassay antibodies were detected in sera of rats immunized to tumor isografts. Chart 3 shows the pattern of pooled sera from rats immunized against syngeneic (WF-anti-W49) and allogeneic (BN-anti-W163) tumors tested on extracts of different colon carcinomas and of other neoplastic or normal tissues. It is feasible to analyze also allogeneic sera as no major histocompatibility antigens are present in this type of extracts. The antibody responses can also be followed during the immunization procedures (chart 4).

Two methods were evaluated for analysis of binding specificities of reactive sera. Before the specificity analysis was performed, the sera were titrated to find the dilution, at which the antibody concentration was

just below excess (chart 5). The method of using the solid phase immunosorption on micro-columns of Sepharose with covalently linked tissue extracts, was mainly suitable for qualitative analysis. By the same volume of serum required for direct testing in the RIA, it was possible by this method to absorb the reactive antibodies effectively with the appropriate extracts. Chart 6 exemplifies this with the same serum (WF-anti-W49) as shown in chart 3. The amount of extract used for immunosorption corresponds to 20 times the amount used as target antigen in the RIA.

As an alternative method for evaluation of specificity competitive binding inhibition was investigated. As an example a specific inhibition curve is presented (chart 7) with total inhibition achieved with 33 $\mu\text{g/ml}$ of colon carcinoma extracts, while no inhibition was detected with normal tissue extracts. A prozone phenomenon with increased binding was detected at low inhibitor antigen concentrations. The amount of antigen needed for inhibition varied greatly among the sera studied. Therefore a two stage analysis was performed by first running a broad titration with a wide range of antigen concentrations, followed by a more detailed analysis of the inhibition curve in the concentration range of interest. By these methods the serum antibodies were shown to be directed to tumor associated antigens shared by DMH-induced colon carcinomas.

DICUSSION

We have recently described an indirect ^{125}I -SpA radio-immunoassay for binding of rat Ig(sub)classes to cell surface histocompatibility antigens on viable attached cells (10,11). The direct application of this method for detection of antibodies against rat colon carcinomas was not sucessful. The reasons for this have not been further evaluated but may include a selection of weakly antigenic variants during explantation and growth of the tumor cells in vitro. This would lead to a low density of antigens in the wells at monolayer growth and consequently to a low sensitivity.

The use of viable target cells in the assay of tumor antigens also poses problems in the analysis of antigenic specificity requiring cultures of various normal tissues and availability of large cell numbers. Therefore, instead of using cells, we have tested solubilized antigens prepared in various ways and now report the successful use of butanol tissue extracts as target antigens in a binding assay of anti-tumor antibodies.

The single-phase 1-butanol extraction procedure described by LeGrue et al (7), was modified by exclusion of the trypsinization step and was found to be a very simple way to obtain antigenic material directly from in vivo grown tumors or from normal tissues. This extraction method is non-cytolytic, which results in diminished contamination with cytoplasmic molecules. As previously reported for other species (8) alloantigens

were not detectable in the extracts making them feasible also for studies of allogeneic tumor immunizations. It is also possible to use crude cell membrane preparations as starting material for the butanol extraction. However, this is a much more laborious technique, but it has the advantage of allowing the use of frozen stored tissue and it can be used for tissues from which it is difficult to obtain cell suspensions of good quality and yield.

The extracts from both tumors and normal tissues contained a detectable amount of immunoglobulins. As the studied tumor is immunogenic in immunocompetent rats, there may be formation of immune complexes at the cell surfaces which may mask the antigens in the subsequent analysis. Therefore the possible advantage of harvesting tumor tissue from nude rats was studied. However, comparable amounts of immunoglobulins were detected in the two types of extracts. It has recently been reported that nude rats can produce antibodies against transplanted tumors (4). Since the amount of Ig in normal tissue extracts is also about the same, which indicates that only a minor part of the Ig present in the tumor tissue may represent specifically bound antibodies, the lack of improvement is probably not due to the fact that nude rats might be capable of producing antitumor antibodies (4). The use of rapidly growing tumors may result in harvesting of the tumor material before any substantial amount of Ig has been produced by the rat. The tumor growth may also suppress the humoral response.

In any solid phase immunoassay the binding capacity of

the solid matrix is a critical experimental parameter. A great problem in using crude preparations as target antigens is competition between the bulk of molecules in the mixture and the molecules of interest during adsorption to the plastic. This often leads to a low density of antigen at saturation of the plastic surface resulting in low sensitivity levels (9). The titration experiments indicate that this is not a problem with the studied antigens in the butanol extracts, as no decrease in binding was found even at high concentrations of the extracts. Therefore, these antigens bind unusually well to the plastic and are not competed out by the bulk of other molecules present. The fact that pH or ion strength do not dramatically influence the adsorption indicates that the antigens bind to the wells by strong hydrophobic interactions.

The first level of specificity analysis is performed by testing the sera in parallel on test and control target antigens. This will distinguish sera reacting with molecules present in both targets or sera with background binding to the plastic. However, if a serum reacts with two tumor targets, another method is required to determine whether this serum contains one population of antibodies reacting with the same molecules in the two targets or contains two independent populations of antibodies reacting with different molecules in the two target extracts. For distinction between these two alternatives, the binding test has to be combined with adsorption or inhibition studies with the different antigens. Two variants of solid phase

immunosorption are possible , the batch technique and the column procedure. The use of columns is more efficient and is useful also for antibodies with relatively low affinity. However, this technique provides mainly qualitative information about the binding specificities of the sera. For quantitative analysis of specificity we used competitive binding inhibition experiments, in which the soluble antigens were titrated for their ability to inhibit the binding of the antibodies to the antigens adsorbed to the plastic wells. This method can also be used to quantitate the relative antigenicity of various antigen preparations and the degree of cross reactivity between different tumor extracts. By use of the column immunosorption technique and the competitive binding inhibition assay, it was possible to demonstrate specificity of the analyzed sera for colon carcinoma associated antigens.

The use of cell extracts provides a continuous and consistent source of antigen that undoubtedly contributes to the reproducibility of the radioimmunoassay. The assay is now being used for studies of the humoral immune response against rat colon carcinoma. The extracts can also be used as starting material for further purification and characterization of the tumor associated antigens.

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CHARTS:

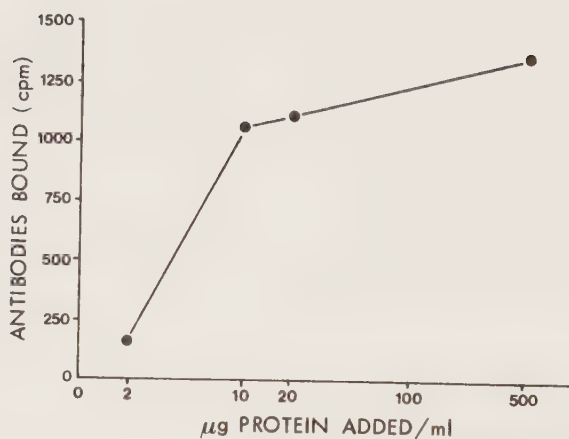


Chart 1:

Binding of IgM of a tumor immune serum (WF-anti W49) to microplate wells containing W49 tumor extracts attached to the plastic by prior incubation with various concentrations of 1-butanol extracts of the tumor. Background binding is subtracted. The serum is in excess.

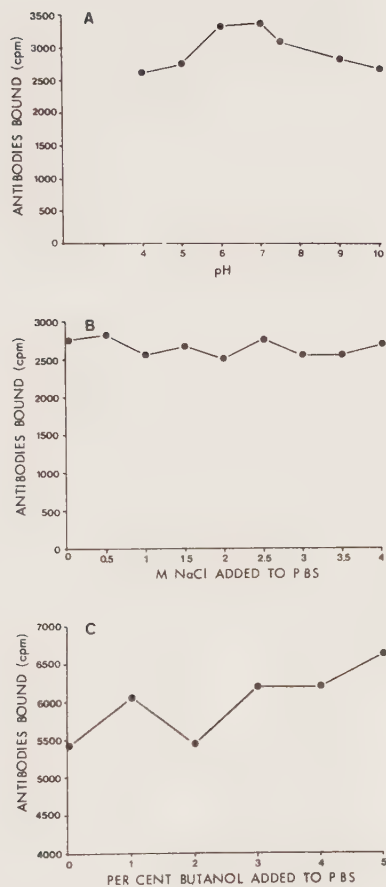


Chart 2:

Influence of various buffer compositions on the adsorption of a butanol extract to the wells. The antigen (W49) and the serum (WF-anti W49) concentrations and other incubation conditions were kept constant. Background is subtracted. (A) influence of pH, (B) influence of NaCl-concentration, (C) influence of 1-butanol concentration.

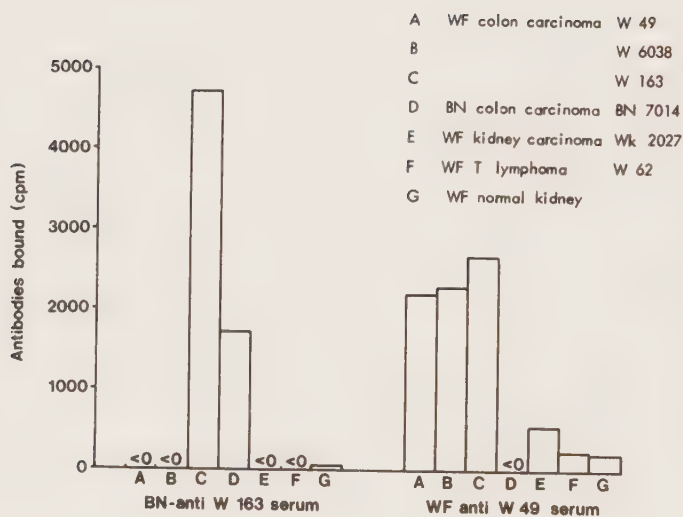


Chart 3:

Binding pattern of IgM of two tumor immune sera (diluted 1/20) on butanol extracts of various tumor- and normal tissues. 1 μ g protein/well corresponds to 0.2 μ l tissue pellet. Background binding is subtracted.

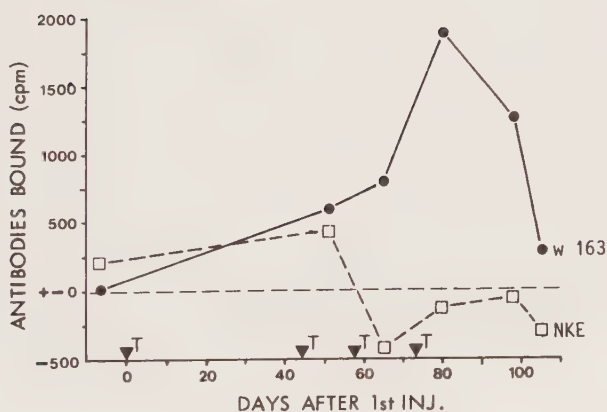


Chart 4:

Binding of IgM of a serum (diluted 1/20) obtained during immunization of a BN-rat with W163 colon carcinoma cells. Background binding is subtracted. (T) injection of 1 million viable W163 cells intraperitoneally. No binding of other Ig(sub)classes was found.

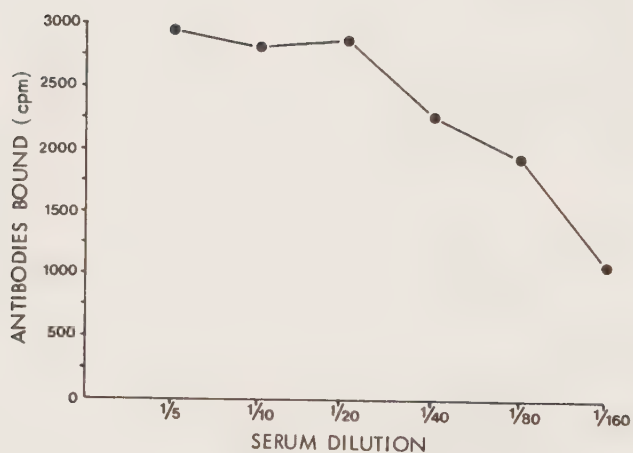


Chart 5:

Titration of a tumor immune serum reactive against W 49-tumor extract. Background binding is subtracted.

A WF colon carcinoma W 49 - sepharose C WF kidney carcinoma Wk 2027 - sepharose
 B WF colon carcinoma W 163 - sepharose D WF normal kidney - sepharose

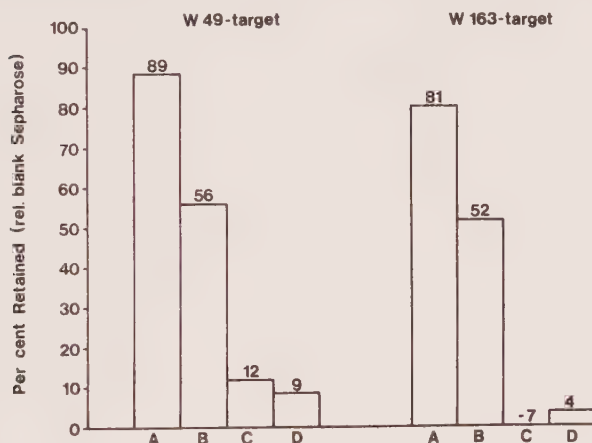


Chart 6:

Immunosorption of IgM of a tumor immune serum (WF anti-W49, diluted 1/20) on columns (0.4 ml) with tumor and normal tissue extracts. The amount of antibody retained by the test columns is expressed in percentage of the amount passing a blank-sepharose column. Background binding is subtracted.

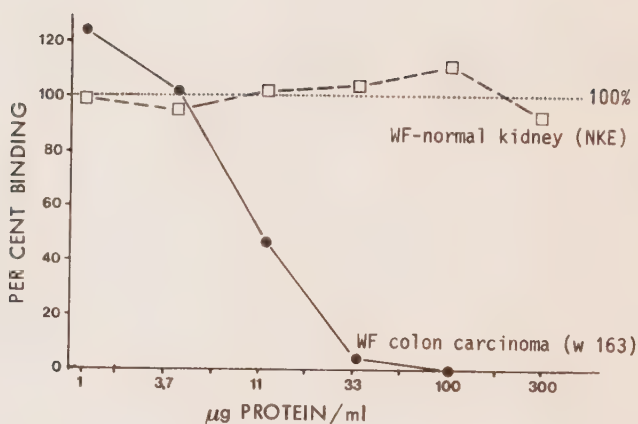


Chart 7:

Competitive binding inhibition experiment with a tumor immune serum (BN anti-W163; IgM) inhibited by a soluble W163-tumor extract and a normal WF kidney tissue extract. Fifty microliters of diluted extracts were mixed with 50 μ l serum diluted 1/10 and added to wells with adsorbed W163-extracts (1 μ g/well). The results are expressed as percentage of the binding activity obtained by admixture of PBS instead of butanol extracts. Background binding is subtracted.

PARTIAL BIOCHEMICAL CHARACTERIZATION OF 1-BUTANOL-
EXTRACTABLE RAT COLON CARCINOMA ANTIGENS DEFINED
BY SYNGENEIC ANTIBODIES.

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ABSTRACT

We have recently demonstrated that tumor antigens can be extracted from rat colon carcinoma tissue by a single-phase solution of 2.5 % 1-butanol. By use of such extracts as target antigens in an indirect ^{125}I -SpA RIA, syngeneic antibodies can be detected, which are directed to tumor associated antigens shared by dimethylhydrazine-induced rat colon carcinomas but not expressed in normal tissues. To characterize the involved tumor antigens pools of tumor antisera with such specificity were used to detect the butanol extractable tumor antigens (BETA) in various extracts preparations or fractions obtained from the crude extracts.

The techniques used for characterization included hydrophobic interaction chromatography, lectin affinity chromatography, ion exchange chromatography and chromatofocusing performed in analytical scale and treatment with proteases. This analysis demonstrated that BETA are a group of acid, hydrophobic non-glycoproteins.

INTRODUCTION

It is now widely accepted that tumor cells frequently express new or altered cell surface molecules and that an anti-tumor immune response may be elicited against these molecules. This immune response may result in rejection but also in growth enhancement of the tumor. The tumor antigens may induce antibody production in animals bearing a syngeneic tumor graft and in animals immunized with tumor material. Different chemically induced tumors have often been shown to contain a multitude of tumor antigens with different immunobiological and molecular properties. Despite numerous attempts to characterize these antigens in different experimental tumor models, very little information is available about their chemical structure. The most detailed biochemical studies of tumor associated antigens have been reviewed by Law et al (5).

Various methods have been used for the solubilization of tumor antigens from the surface of tumor cells. Extraction of tumor antigens with a single-phase solution of 2.5 % 1-butanol was initially described by LeGrue et al (6). They showed that tumor-specific transplantation antigens of a murine 3-methylcholanthrene-induced fibrosarcoma could be detected in this extract by the use of an immunoprotection test. Partial purification by preparative isoelectric focusing revealed that most of the antigen focused between pH 6.1 and pH 6.6. Low molecular weight macromolecules (12 - 14

Kdalton) obtained by gel filtration chromatography of crude butanol extracts of a murine mammary adenocarcinoma, were found to be active in vitro in a leukocyte adherence inhibition (LAI) assay and in vivo in an immunoprotection assay (3). In studies on butanol extracts from the murine Bl6 melanoma cell line, LeGrue (7) showed that the membrane components removed by 1-butanol can functionally reassociate with viable cells previously subjected to butanol-treatment, leading to the reexpression of the original phenotype. By serological techniques human tumor associated antigens have also been detected in butanol extracts of human sarcomas (9).

Serological assays allow rapid detection of antigen following chromatographic fractionations or exposure of complex tumor extracts to other analytic procedures. We recently reported that butanol extracts of rat colon carcinoma tissues can be used in a radio immuno assay for analysis of tumor-reactive antibodies in syngeneic rat sera (8). This assay is used in the present investigation of the biochemical characteristics of the rat colon carcinoma associated antigens in conjunction with various analytical chromatographic procedures. The obtained information will create a basis for further purification at a larger scale and will be important in the continued analysis of the biochemical and immunobiological properties of the antigens involved.

MATERIAL AND METHODS

Animals, tumors and serum:

Rats of the strains Wistar / Furth (WF) and Brown Norway (BN) were used as tumor and serum donors. They were maintained by continuous, single-line brother and sister mating. The rats were kept on a standard pellet diet and water ad libitum.

Colon adenocarcinomas (W49 and W163) induced with 1,2-dimethylhydrazine (DMH) were kept in serial intraperitoneal passage in WF rats.

Sera were obtained from rats immunized against W49 or W163 colon carcinoma. Syngeneic WF anti-W49 sera were collected from WF rats sensitized with irradiated W49-cells and temporary growth of the same tumor. Allogeneic BN anti-W163 sera were obtained by three biweekly immunizations with viable nonirradiated W163 cells. Serum was collected from individual rats and tested for antitumor reactivity in the radio immuno assay. Positive sera of the respective reactivity were pooled. By use of the recently described specificity tests (8) they were shown to be specific for 1-butanol-extractable tumor associated antigens shared by DMH-induced rat colon carcinomas.

Pooled sera of normal, untreated inbred WF female and male rats of various ages were used as normal reference serum (NRS) in the assays. Rats were bled under ether

anesthesia by cardiac puncture (for serum pools) or by capillary drainage from the retro-orbital sinus (for individual sera). Sera were separated and stored at - 20°C.

Preparation of crude antigen extracts

Extraction of rat colon carcinoma associated antigens by single-phase solution of 1-butanol was performed as recently described (8). Briefly, tumor tissues or control tissues were minced and pressed through a stainless steel screen and washed four times with about ten volumes of PBS (calcium- and magnesium-free phosphate buffered saline pH 7.3). The tissue fragments were extracted with 2.5% (v/v) 1-butanol (Merck) in PBS for 5 min at room temperature and then centrifuged at 500 x g for 10 min. The supernatant was dialyzed against PBS and after another centrifugation at 40 000 g for 60 min, it was stored at -80°C and was designated as crude butanol extract (CBE). The extracts have been stored for up to seven months without detectable decrease in reactivity. Protein concentrations were determined by the use of the Coomassie Brilliant Blue technique (BioRad laboratories; (1)) using ovalbumin as a standard.

Immunodetection of butanol extracted tumor antigens

(BETA):

The presence of butanol extractable tumor antigens (BETA) in various crude or fractionated butanol tissue extracts was detected by the use of a previously described radio immuno assay for studies on rat colon

carcinoma associated antigens and antibodies (8). Fifty μ l of the material to be tested were incubated for 18h at 4°C in 8-well flat-bottomed stripes (Dynatech Immulon removastripes no M1798A; Laboratorie Design AB, Stockholm, Sweden) placed in 96-well holders (Dynatech M73A) to receive the standard 96-well microtest plate format. After two washes with 200 μ l PBS, the protein-reactive sites on the plastic surface were blocked by incubation for one hour at room temperature (RT) with 200 μ l coating / dilution buffer (CD-buffer; 20% fetal calf serum, 10mM sodium phosphate, 10mM Hepes, 0.15M sodium chloride, 5mM sodium azide, pH 7.30). The CD-buffer was aspirated and 50 μ l of a rat serum pool, known to be specific for BETA, diluted in CD-buffer were added. After incubation for one hour at RT, the sera were aspirated and the plates were washed twice with 200 μ l washing solution (WB-buffer; 10mg/ml BSA (Sigma A-4503), 10mM sodium phosphate, 10mM Hepes, 0.15M sodium chloride, 5mM sodium azide, pH 7.3). Rabbit antiserum to the reactive rat Ig(sub)class was added in volumes of 50 μ l diluted in CD-buffer and the plates were again incubated for one hour at RT and washed twice with 200 μ l WB-buffer. Fifty μ l of Protein-A (SpA), labelled with 125 I by use of the Bolton-Hunter method (4) and diluted in CD-buffer, were added and incubated for one hour at RT. After two washes the bound radioactivity was counted in a gamma counter (Auto-gamma 500C, Packard Instruments) for one minute per well. Each sample was run in duplicate or triplicate and always compared to the binding of the serum pool to unfractionated crude

tumor extract and control extract.

Protease treatment of CBE

Crude butanol extract was adsorbed to the test wells (50 μ l/well, 20 μ g/ml) by incubation at 4°C for 18h. The extract was aspirated and 200 μ l enzyme solution was added per well and incubated at 37°C for 1h. After two washes with PBS, remaining antigenicity was analyzed by the standard radio immuno assay. Enzymes used were 1 mg/ml of trypsin (EC 3.4.21.4; SBL, Sweden) and 4.8 mg/ml of dispase (EC 3.4.24.4; grade II; Boehringer Mannheim) dissolved in PBS pH 7.3.

Screening for binding of BETA to various lectins

Various lectins, covering a broad spectrum of carbohydrate specificities, coupled to solid matrices were used for adsorption of the crude butanol extract. Con A-Sepharose 4B, LcH-Sepharose 4B, WGL-Sepharose 6MB, HP-Sepharose 6MB, and Sepharose 4B Cl were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and LPA-agarose, UEA-I-agarose, RCA-I-agarose, and PNA-agarose were obtained from E.Y Laboratories (Kemila, Stockholm, Sweden). The designations and sugar specificities of the different lectins are summarized in table 1. The lectin-matrices were packed into micro-columns (5x20 mm, 0.4 ml) and equilibrated with PBS before 70 μ l undiluted extract (1.0 mg/ml) were applied. After incubation for 15 min the columns were washed with 1.4 ml PBS at about 3 ml/h. The effluents were collected and analyzed in the radio immuno assay for presence of BETA. The whole procedure was performed at room temperature. The

antigenic reactivities of lectin-adsorbed extracts are compared with the reactivity of extract adsorbed on a column with blank-Sepharose 4B CL.

Hydrophobic interaction chromatography

Phenyl- and Octyl-Sepharose CL-4B (Pharmacia Fine Chemicals) were packed into micro-columns (5x20 mm, 0.4 ml) and equilibrated with PBS. Seventy μ l of extract (1.0 mg/ml) were applied and incubated for 15 min before elution with 1.4 ml of PBS. For one group of columns, 4M sodium chloride was supplemented to the PBS and the extracts. The effluents from Octyl- and Phenyl-Sepharose were compared with the effluents from blank-Sepharose CL-4B obtained under the same conditions.

Twelve ml of butanol-extract (0.5 mg/ml) supplemented with 4M NaCl were applied on a column with 5 ml phenyl-Sepharose (9x80 mm) equilibrated with 5mM sodium phosphate, 4M NaCl pH 7.3. When the unbound material had been washed out with this buffer, the bound fraction was eluted with a 50 ml linear gradient from 4.0 to 0.0M NaCl in 5mM sodium phosphate pH 7.3. At the end of the gradient, the column was eluted with distilled water. Fractions of 3 ml were collected during the separation.

The adsorbed and fractionated extract was analyzed for presence of BETA by use of the radio immuno assay.

Ion exchange chromatography

Ion exchange chromatography experiments were performed

at room temperature with the Fast Protein Liquid Chromatography (FPLC) instrumentation from Pharmacia Fine Chemicals including a Liquid Chromatography Controller (LCC-500), high precision pumps (P-500), UV-monitor with high resolution flow cell (UV-1, HR10), fraction collector (Frac-100), peristaltic pump (P-1), and motor valves (MV-7, MV-8).

A volume of 500 μ l (1.0 mg/ml) crude extract was applied on an anion exchange column (Mono Q HR5/5; 0.5 x 5 cm, 1 ml) equilibrated with 20mM L-histidine pH 6.0. The column was eluted with 2.0 ml of this buffer before elution with a 20 ml linear gradient from 0.0 to 1.0M NaCl in 20mM histidine pH 6.0, followed by 2.0 ml of 20mM histidine, 1.0M NaCl pH 6.0. The separation was performed at 1.00 ml/min and 1-ml fractions were collected.

The presence of BETA in the fractions obtained, were determined by use of the radio immuno assay.

Chromatofocusing of CBE

Crude butanol extract was separated by chromatofocusing between pH 6 and 4 by use of the FPLC-equipment.

A volume of 4.0 ml extract (1.0 mg/ml) was applied on a polybuffer exchange column (Mono P HR 5/20; 0.5 x 20 cm, 4 ml) equilibrated with 25mM bis-TRIS-HCl pH 6.30 (bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane, Sigma B-9754).

Unbound material was eluted with 4.0 ml of this buffer,

before the bound material was eluted with 40 ml of the pH-gradient generating buffer (Polybuffer 74, 7.5 μ mol/pH-unit/ml pH 4.00; Pharmacia Fine Chemicals), followed by 1.0 ml of 25 mM Tris/HCl, 2.0 M NaCl pH 6.3. The fractionation was performed at room temperature at 1.00 ml/min and 1-ml fractions were collected.

RESULTS

Butanol extractable tumor antigens (BETA) were obtained from in vivo grown tumor tissue by a single-phase solution of 2.5% butanol. BETA were detected, in crude or fractionated extracts, in a radio immuno assay by use of syngeneic or allogeneic anti-tumor-serum pools. These serum pools have been shown to be specific for tumor associated antigens shared by DMH-induced rat colon carcinomas but not expressed by normal tissues, when analyzed in specificity tests recently described (8).

In order to explore whether protein was involved in the antigenic structures, tumor extracts adsorbed to test wells were subjected to proteolytic treatment before assay. A decreased amount of bound antibody was obtained after both trypsin (90%) and dispase (61%) treatments indicating that the antigens consist of proteins or are associated with proteins.

The affinity of BETA for various lectins was evaluated by adsorption of butanol extracts on small columns with lectins covalently linked to agarose. As summarized in table 1, no binding to the lectins could be detected although the most widely used lectins, covering a broad spectrum of sugar specificities, were used. Since the antigens do not appear to contain any of the widely distributed sugar moieties it seems that they are not glycoproteins.

As the BETA are extracted from the cell membrane, it may be assumed that they will have hydrophobic properties. This was first studied by adsorption of butanol extracts on microcolumns with phenyl- and octyl-Sepharose. For some columns the ion strength was increased by addition of 4M NaCl to the PBS, as this will favour the hydrophobic interactions. The BETA had high affinity for octyl-Sepharose at both ion strengths, while the binding was lower to phenyl-Sepharose when PBS without 4M NaCl was used (chart 1). As the strong binding to octyl-Sepharose makes elution of bound proteins more troublesome, phenyl-Sepharose was used in the subsequent fractionations. When the butanol extract, applied to phenyl-Sepharose at 4M NaCl, was eluted with a linear salt gradient the antigen was shown to be heterogeneous and was eluted as two separated peaks (chart 2). The two serum pools used detect different antigens. WF-anti-W49 serum detects antigens shared by different colon carcinomas, while BN-anti-W163 serum detects an antigen only detectable on W163 extracts. The presence of BETA in the effluent may indicate that the column was overloaded, as nearly all BETA (91%) have been shown to bind to phenyl-Sepharose in 4M NaCl when tested on micro-columns where the phenyl-Sepharose is in large excess.

After pilot experiments with batch-incubations of CBE in tubes with different ion exchangers and at varying pH, the extracts were subjected to Mono-Q anion exchange chromatography at pH 6.0 (chart 3). At this pH most of the proteins (92%) bound to the column. Bound material

was eluted by a linear NaCl gradient. The majority of the BETA-reactivity appeared in the fractions eluted between 0.3 and 0.5M NaCl, containing about 10% of the UV-absorbing material and eight detectable peaks on the chromatogram. A small amount of BETA was detected at the start of the gradient, indicating a fraction of very slightly retarded antigens.

The crude butanol extract was chromatofocused between pH 6 and pH 4, by use of the Mono-P polybuffer exchanger and polybuffer 74. All of the BETA bound at pH 6.30, but could not be eluted with polybuffer ranging to pH 4.0. This pH is the lower limit for chromatofocusing with this system. The BETA could be detected, however, in the 2M NaCl eluate following the pH-gradient elution (chart 4). This indicates that BETA belong to a group of acid proteins with pI below 4.0.

DISCUSSION

We have recently demonstrated that soluble tumor associated antigens, extracted from tumor tissue using a single phase solution of 2.5% 1-butanol, can be used in an indirect ^{125}I -SpA radio immuno assay for detection of humoral antibodies to syngeneic rat colon carcinomas. The establishment of two types of specificity tests, solid phase immunosorption and competitive binding inhibition, for antibodies binding to tumor extracts were also described (8). With these techniques tumor-reactive antibodies are detected in sera of rats immunized to tumor isografts. The specificity analysis shows that these antibodies are directed to tumor associated antigens shared by DMH-induced rat colon carcinomas but not expressed in normal tissues. Sera with such specificity were pooled, and these pools were used to follow the presence of the butanol-extractable tumor antigens (BETA) during various treatment and fractionation procedures.

The fact that extracts could be used in a wide range of concentrations for adsorption to the test wells, and the relative independence of the adsorption on pH and ion strength of the adsorption buffer, make the detection of BETA relatively easy, as no titrations or laborious buffer changes are needed before analysis of the different fractions. However, it should be stressed that this mode of testing provides mainly qualitative or semi-quantitative results. For a real quantitation of

BETA-concentrations, the competitive binding radio immunoassay has to be used. This will require buffer changes and a larger amount of sample. It is a great advantage that BETA are soluble in PBS without the use of detergent, as detergents would introduce difficulties in the performance of several of the analyses described in this communication.

An important first step in the characterization of the tumor antigens is to investigate whether the molecules are proteins or glycolipids, as this will greatly influence the selection of methods for further characterization. The effect of two proteases with different specificities, trypsin and dispase, on crude butanol extracts indicates that BETA are proteins or are associated with proteins.

Opposite to what has been reported for several other cell membrane proteins, the BETA show no affinity for the lectins tested. It thus appears that the BETA do not include glycoprotein with any of the widely distributed sugar moieties. An increased reactivity was observed after absorption with some of the lectins. This probably depends on removal of some glycoproteins which otherwise would interfere with the BETA during adsorption to the plastic. Full scale chromatography on ConA- and HP-Sepharose excluded the possibility of a prozone phenomena in the binding of BETA to the lectins, as no binding could be detected (data not shown). Lectin affinity chromatography of butanol extracts thus provides a suitable negative selection technique for

BETA purification.

Although hydrophobic interaction chromatography should be expected to be useful in studies of molecules extracted from cell membranes, no reports have previously appeared using this method for characterization and purification of tumor associated membrane antigens. In our studies we found the use of hydrophobic interaction chromatography very successful for purification of butanol extracted tumor antigens. The majority of the proteins in the crude extract does not bind to phenyl-Sepharose at 4M NaCl but it is demonstrated that BETA do and it is further shown that it can be eluted by decreased ion strength. Elution with a linear descending salt gradient indicates a heterogeneity in hydrophobicity of the BETA, as they elute in two well separated peaks. These experiments show that the use of hydrophobic interaction chromatography may be effective in the purification of membrane molecules, comparable to many affinity chromatography methods.

Ion exchange chromatography may also be an effective procedure for purification of the BETA. The antigenic material was detected in fractions that include only about 10% of the total UV-absorbing material. The separation may, however, be further improved by running the anion exchange chromatography at pH 4.0 instead of pH 6.0, as only a minor fraction of the proteins will bind to the column at pH 4.0.

Chromatofocusing experiments were performed in order to estimate the pI of the BETA. The BETA bind to the polybuffer exchange columns, but are not eluted at pH 4.00, which is the low pH limit for this method. This shows that BETA belong to a group of acid proteins, but the exact pI and the heterogeneity according to pI can not be established by this method. Such acid proteins are rather unusual and according to a recent survey less than 5% of the proteins analysed have a pI below pH 4.0 (2). However, this survey includes mainly soluble proteins and it is therefore quite possible that membrane proteins have a different distribution of pI.

Attempts have been made to estimate the molecular weights of the BETA by use of SDS-polyacrylamide electrophoresis followed by slicing of the gel rods. However, the immunochemical detection was complicated by the presence of SDS, which influenced the adsorption of BETA to the test wells. Immunoblotting or immunoprecipitation techniques are being established to improve this analysis.

In conclusion, this preliminary characterization have shown that rat colon carcinoma associated antigens, extractable with 1-butanol, belong to a group of hydrophobic, acid proteins that do not contain any widely distributed sugar moiety. These data will function as a basis for further purification and characterization of the antigens, and will be important in the continued studies of their biochemical and

immunobiological properties. The results strongly indicate that a combination of hydrophobic interaction chromatography on phenyl-Sepharose and ion exchange chromatography on Mono Q anion exchanger at pH 4.0 will result in a high degree of purification. This combination will make a rapid separation of a large amount of material feasible. It may also be possible to use the recently available hydrophobic interaction FPLC-columns (Pro-RPC HR 5/10, Pharmacia Fine Chemicals) instead of phenyl-Sepharose. This would have the advantage that the combined separation could be performed in 1-2 h.

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Table 1. Screening for affinity of butanol-extracted tumor antigens (BETA) to different lectins.

COLUMNS	SUGAR SPECIFICITY	% BINDING REL.* BLANK-SEPHAROSE	AFFINITY FOR THE LECTIN
Unseparated control	-	103	
Blank-sepharose	-	100	
Wheat germ lectin (WGL)	(β (1-4) D-GlcNAc) ₂	99	-
Helix pomatia (HP)	α -D-GalNAc	156	-
Concavalina ensi- formis (ConA)	α -D-Glc; α -D-Man	177	-
Limulus polyphemus (LPA)	Sialic acid	106	-
Ulex europeus (UEA-I)	α -L-Fucose	124	-
Ricinus communis (RCA-I)	α -D-Gal	98	-
Lens culinaris (LcH)	α -D-Glc, α -D-Man	104	-
Peanut lectin (PNA)	Gal- β (1-3)-GalNAc	87	-

* Crude butanol extract of W49-tumor is passed through microcolumns with lectin-agarose before adsorption to the microtest wells. The presence of antigenic material is detected by the syngeneic anti-W49 serum pool. The binding to adsorbed extract is expressed as percentage of the binding obtained with extract absorbed on blank-sepharose.

CHARTS

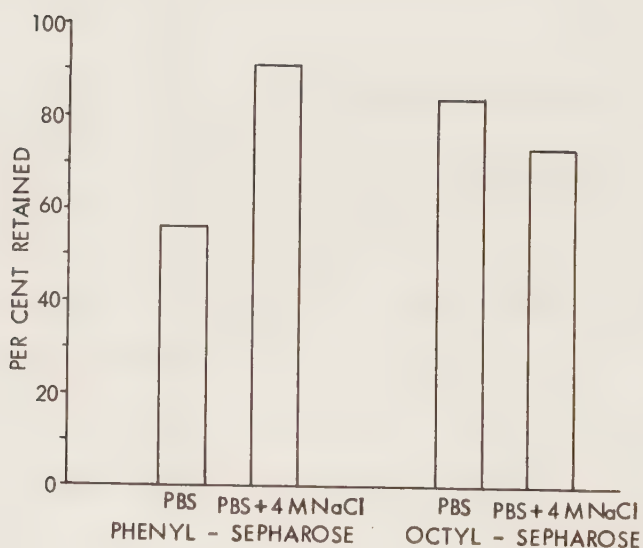


Chart 1:

A crude butanol extract of W49 colon carcinoma was passed through hydrophobic interaction chromatography columns before adsorption to the microtest wells. The presence of antigenic material was detected by a syngeneic WF-anti-W49 serum pool. The amount of antigen retained by the test columns is expressed in percentage of the amount passing a blank-Sepharose column. Background binding is subtracted.

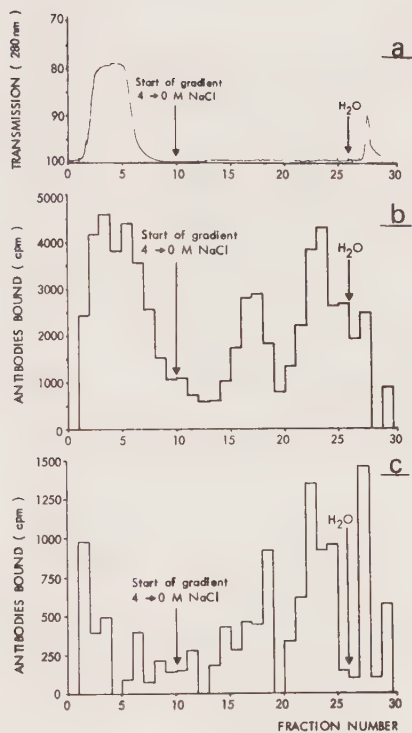


Chart 2:

Hydrophobic interaction chromatography of a crude butanol extract of W163 colon carcinoma on phenyl-Sepharose. The sample was applied in 4M NaCl. The bound material was eluted by a linear gradient between 4.0 M and 0.0 M NaCl, followed by distilled water (a). The presence of BETA in the fractions was analyzed in a RIA with an allogeneic BN-anti-W163 serum pool (b) and a syngeneic WF-anti-W49 serum pool (c). These two serum pools detect different tumor associated antigens. Background binding is subtracted.

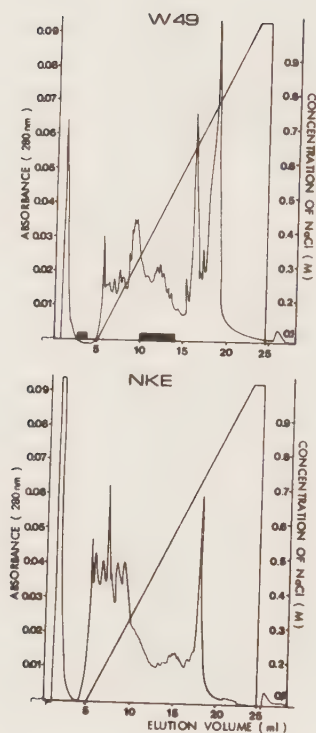


Chart 3:

Ion exchange chromatography of crude butanol extract of W49 colon carcinoma (W49) and normal kidney (NKE) on Mono-Q anion exchange column. The bound material was eluted by a linear salt gradient between 0.0 M and 1.0 M NaCl. The presence of BETA in the collected 1-ml fractions was detected by a syngeneic WF-anti-W49 serum pool in a RIA with adsorbed fractions as target antigen. The bars show fractions where BETA were detected.

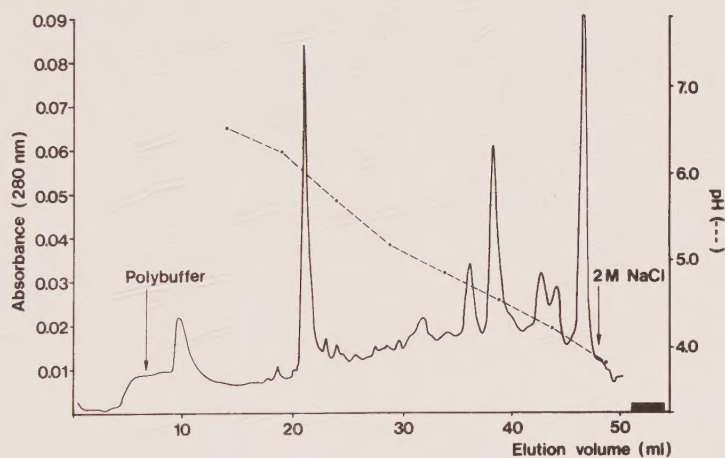


Chart 4:

Chromatofocusing of crude butanol extract of W49 colon carcinoma on a Mono-P polybuffer exchange column. The bound material was eluted by a linear pH-gradient between pH 6 and pH 4 which was generated by elution with polybuffer 74 pH 4.0, followed by elution with 2 M NaCl. The presence of BETA in the collected 1-ml fractions was analyzed by a syngeneic WF-anti-W49 serum pool. The bar shows the fractions in which BETA were detected. The pH of the collected fractions was measured with a standard pH-meter.

